The effect of alternative forage species and gibberellic acid on nitrate leaching

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by

Roshean Rose Woods

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Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy.

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Nitrogen (N) leaching from soil is a significant concern for intensively grazed forage-based systems because it can cause a decline in water quality. Urine patches are the main source of N leaching in these systems. In New Zealand, increasing pressure to increase export earnings, while reducing N leaching loss from agriculture, poses a challenge for farmers and mitigation options are urgently needed. One approach is to increase the uptake of urine-N by forage plants, thereby reducing N leaching. The aim of this PhD programme was to increase our knowledge and understanding of the effects of alternative forages and gibberellic acid (GA) on N leaching from grazed agricultural soil.

Three lysimeter experiments were conducted. The first quantified the effect of forage type (perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) (RGWC), Italian ryegrass (*Lolium multiflorum* Lam.), and lucerne (*Medicago sativa* L.)) and GA application on N leaching, herbage dry matter (DM) yield, and N uptake. Lysimeters (0.5 m diam., 0.7 m deep) were treated with urine (700 kg N ha^{-1}), and ± GA (8 g GA ha^{-1}) in May 2014. A ^{15}N balance was conducted to determine the fate of the applied urine-N. The second lysimeter experiment determined the N leaching loss, herbage DM yield, and N uptake from the urine patch of an Italian ryegrass, plantain (*Plantago lanceolata* L.), white clover mixture (Italian-Plantain Mix), compared with RGWC. Urine was applied in March 2015 and included a 700 kg N ha^{-1} (Urine 700) treatment, and urine-N at the concentration excreted by cows grazing each forage type (Urine Actual). The third experiment used smaller lysimeters (0.18 m diam., 0.5 m deep) to determine the N leaching loss, herbage DM yield, and N uptake response of RGWC to an application of GA (8 g GA ha^{-1}) over a range of urine-N rates (0, 25, 50, 100, 200, 400, and 700 kg N ha^{-1}) applied in April 2016.

A pot experiment was also conducted to determine whether there were any differences in the soil ammonia-oxidising bacteria and archaea beneath perennial ryegrass and Italian ryegrass, these were

compared with bare soil. Pots (0.0144 m^2, 0.13 m deep) were destructively harvested 1, 15, 30, 61, and 90 days following urine application (700 kg N ha^{-1}) in May 2015.

Significant reductions in N leaching loss, were shown for Italian ryegrass (35%) and Italian-Plantain Mix (45-89%) forages, when compared with RGWC. The mechanisms behind this were a reduction in urine-N excretion (for Italian-Plantain Mix), and increased cool-season uptake of urine-N by the Italian ryegrass. This was reinforced when no difference in soil ammonia-oxidisers was shown between perennial ryegrass and Italian ryegrass which suggested that Italian ryegrass was not inhibiting nitrification. Gibberellic acid had no effect on N leaching. This research has clearly shown Italian ryegrass, and Italian-Plantain Mix as promising alternatives to RGWC, which could reduce N leaching losses from grazed systems. Lucerne is not recommended as an alternative to RGWC, as N leaching losses were higher under grazed conditions. Gibberellic acid had no direct effect on N leaching loss and so is not recommended as a direct mitigation tool for N leaching losses in grazed systems.

Keywords: nitrogen, mitigation, pasture, lysimeters, perennial ryegrass (*Lolium perenne*), white clover (*Trifolium repens*), Italian ryegrass (*Lolium multiflorum*), lucerne (*Medicago sativa*), plantain (*Plantago lanceolata*), herbage N uptake, grazed forages, animal urine, ^{15}N isotope, N balance.

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- Woods, R. R., Cameron, K. C., Edwards, G. R., Di, H. J., Clough, T. J., 2016. Does gibberellic acid reduce nitrate leaching losses from animal urine patches? In: Integrated nutrient and water management for sustainable farming. (Eds L.D. Currie and R. Singh). http://flrc.massey.ac.nz/publications.html. Occasional Report No. 29. Fertilizer and Lime Research Centre, Massey University, 9-11 February 2016, Palmerston North, New Zealand. 5 pages.
- Woods, R. R., Cameron, K. C., Edwards, G. R., Di, H. J., Clough, T. J., 2016. How could plants help us to reduce nitrogen losses from dairy farms? New Zealand Society of Soil Science and Soil Science Australia joint conference: Soil, a Balancing Act Downunder, 12-16 December 2016, Queenstown, New Zealand.

Some of the results of this project have been published in:

- Woods, R. R., Cameron, K. C., Edwards, G. R., Di, H. J., & Clough, T. J. 2016. Effects of forage type and gibberellic acid on nitrate leaching losses. *Soil Use and Management, 32*(4), 565-572. doi:10.1111/sum.12297
- Woods, R. R., Cameron, K. C., Edwards, G. R., Di, H. J., & Clough, T. J. 2017. ^{15}N recoveries from ruminant urine patches on three forage types. *Plant and Soil*, 1-13. doi:10.1007/s11104-017-3270-5

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Acronyms and Abbreviations

^{15}N	Nitrogen-15 isotope	ME	Metabolisable energy
AMO	Ammonia monooxygenase	Mg	Magnesium
amoA	Ammonia monooxygenase A gene	$MgCl_2$	Magnesium chloride
ANOVA	Analysis of variance	MgO	Magnesium oxide
AOA	Ammonia-oxidising archaea	MP-AES	Microwave plasma atomic emission spectroscopy
AOB	Ammonia-oxidising bacteria	MS	Milksolids
BNI	Biological nitrification inhibition	N	Nitrogen
BS	Base saturation	N_2	Dinitrogen
C	Carbon	N_2O	Nitrous oxide
Ca	Calcium	Na	Sodium
$CaCl_2$	Calcium chloride	$NaHCO_3$	Sodium bicarbonate
$CaSO_4$	Calcium sulphate	NED	N-(1-Naphthyl) ethylenediamine Dihydrochloride
cDNA	Complementary deoxyribonucleic acid	NH_2OH	Hydroxylamine
CEC	Cation exchange capacity	NH_3	Ammonia
CO_2	Carbon dioxide	NH_4^+	Ammonium
CP	Crude protein	NH_4SO_4	Ammonium sulphate
CTAB	hexadecyltrimethylammonium bromide	NIRS	Near infra-red spectroscopy
DI	Deionised	NIWA	National Institute of Water and Atmospheric Research
DM	Dry matter	NO_2^-	Nitrite
DMD	Dry matter digestibility	NO_3^-	Nitrate
DNA	Deoxyribonucleic acid	NS	Nonsignificant
DW	Dry weight	NZ	New Zealand
EF	Emission factor	O_2	Oxygen
FIA	Flow injection analysis	OTCR	Open tubular cadmium reactor
FW	Fresh weight	P	Phosphorus
GA	Gibberellic acid	PCR	Polymerase chain reaction
HAO	Hydroxylamine oxidoreductase	PEG	Polyethylene glycol
HCl	Hydrogen chloride	qPCR	Quantitative polymerase chain reaction
ICP-OES	Inductively coupled plasma optical emission spectrometry	PVC	Polyvinyl chloride
IRMS	Isotope ratio mass spectrometry	RG	Ryegrass
K	Potassium	RGWC	Perennial ryegrass-white clover
KCl	Potassium chloride	RNA	Ribonucleic acid
KH_2PO_4	Potassium dihydrogen phosphate	S	Sulphur
$KHSO_4$	Potassium bisulphate	TBE	Tris/Borate/Ethylenediaminetetraacetic acid
LB	Lysogeny broth	USDA	United States Department of Agriculture
LIC	Livestock Improvement Corporation	WHO	World Health Organisation
LSD	Least significant difference	WSC	Water soluble carbohydrates

Chapter 1

Introduction

1.1 General Introduction

Nitrate (NO_3^-) leaching from soil into water is a significant environmental concern in intensively grazed New Zealand forage-based systems, but also represents a loss of soil fertility. Elevated levels of nitrate in surface waters can cause eutrophication, a process by which high nutrient levels cause algal blooms and excessive plant growth which consume oxygen causing other aquatic life to die (Howarth, 1988; Smith & Schindler, 2009). This represents a significant decline in water quality. If nitrate is leached into drinking water supplies, this is considered a danger to human health when concentrations exceed 11.3 mg NO_3^--N L^{-1} (equal to 50 mg NO_3^- L^{-1}) (WHO, 2011). The primary consequence of high nitrate levels in drinking water is methaemoglobinaemia in babies (blue baby syndrome) (WHO, 2011). Livestock are also at risk of methaemoglobinaemia, and abortions in cattle can occur when drinking water is high in nitrate; concentrations of 40-100 mg NO_3^--N L^{-1} are considered a risk (Di & Cameron, 2002). Nitrate leaching processes, factors affecting NO_3^- losses and methods to reduce nitrate leaching have been thoroughly reviewed by Cameron *et al.* (2013).

In 2014, the New Zealand Government issued a National Policy Statement on Freshwater Management (Ministry for the Environment, 2014). Under this policy, Regional Councils must ensure that freshwater quality standards are met in rivers and lakes within their region. Thus Regional Councils are currently putting together Land and Water Plans in order to help meet their requirements under this policy. Nitrogen is specifically mentioned in regional plans because in many areas, N loads in some water bodies are higher than what is considered sustainable. Limits on N leaching loss are being imposed in many catchments already via the Land and Water Plans. For consents to be granted, many farmers will be required to reduce the amount of N leaching from their property below their current levels.

Due to the increased public and government pressure to reduce nitrate leaching, it is necessary to develop and test new mitigation options which farmers could use to reduce nitrate leaching in New Zealand grazed forage systems. One approach is to increase the uptake of N by the forage, particularly during the cooler seasons when there is the highest risk of leaching. If plants can more efficiently utilise N (e.g. from concentrated urine patches) at these times of year, this may reduce the amount of NO_3^- which is leached from the soil into drainage water. Benefits of this system may also occur through increased production due to higher N use efficiency. Another approach that has been suggested is to

use gibberellic acid to stimulate plant growth and plant N uptake, thus reducing the risk of NO_3^- leaching.

1.2 Aims and Objectives

The aim of this PhD programme is to increase our knowledge and understanding of the effects of alternative forages and gibberellic acid on NO_3^- leaching from grazed agricultural soils.

The research program had the following objectives:

Objective 1: To quantify the effect that forage N uptake has on N leaching from urine applied to a range of different forage types.

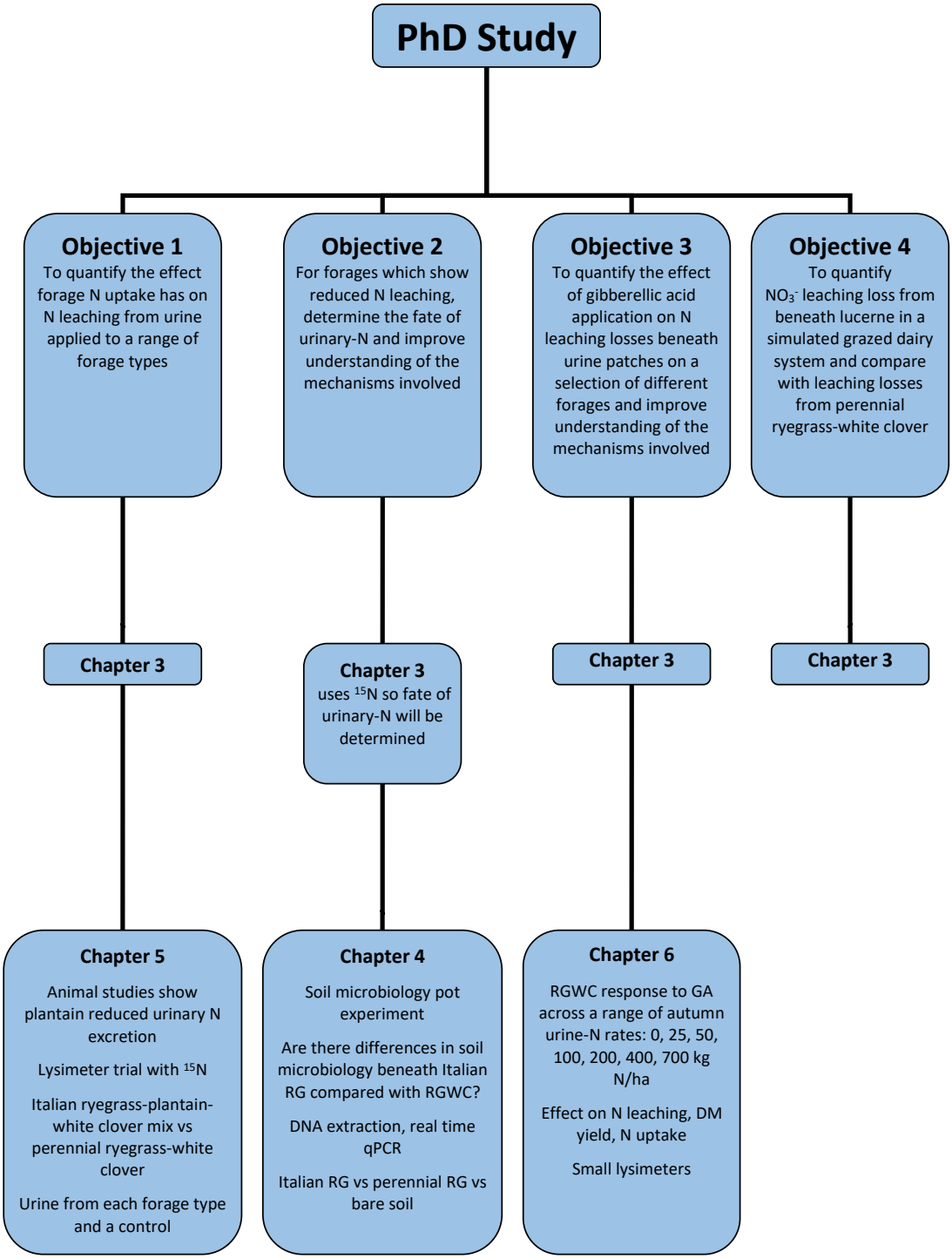
Objective 2: For forages which show reduced N leaching, determine the fate of urinary-N and improve understanding of the mechanisms involved.

Objective 3: To quantify the effect of gibberellic acid application on N leaching losses beneath urine patches on a selection of different forages and improve understanding of the mechanisms involved.

Objective 4: To quantify NO_3^- leaching loss from beneath lucerne in a simulated grazed dairy system and compare with leaching losses from perennial ryegrass-white clover.

Three field experiments using soil monolith lysimeters, and one pot experiment were conducted to pursue these objectives.

This thesis contains seven chapters. Chapter 1 provides a general introduction. Chapter 2 reviews the literature, identifies gaps in existing knowledge of the effects of alternative forages and gibberellic acid on N leaching, and establishes the research hypotheses. Chapters 3, 4, 5, and 6 are experimental chapters. Chapter 7 includes an evaluation of the hypotheses, conclusions, and recommendations for future research.


PhD Study
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To quantify NO_3^- leaching loss from beneath lucerne in a simulated grazed dairy system and compare with leaching losses from perennial ryegrass-white clover
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Chapter 3
uses ^{15}N so fate of urinary-N will be determined
Chapter 3
Chapter 3
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Chapter 6
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Effect on N leaching, DM yield, N uptake
Small lysimeters

Chapter 2

Literature Review

2.1 Introduction

The purpose of this review was to evaluate and synthesise the literature in order to highlight gaps in the current knowledge. This review covers:

- An introduction to the New Zealand dairy industry and problems associated with grazed forage systems, including urine deposition.
- Nitrogen cycling in agricultural systems, particularly covering the fate of urine-N relating to N leaching.
- Current state of our knowledge of alternative forages with the potential to reduce N leaching losses from grazed agricultural systems.
- Current state of our knowledge of gibberellic acid as a potential mitigation option for N leaching from grazed agricultural systems.
- A review of ^{15}N balance studies determining the fate of urine-N in grazed forage systems.

The knowledge gaps identified in this literature review were used to formulate the research objectives and hypotheses.

2.2 Current knowledge of N cycling in agricultural systems

2.2.1 The New Zealand dairy industry

In New Zealand, pastoral dairy farming is based on cows sustaining a high level of milk production over a long (~270 day) lactation. This is dependent on feed supply, and N fertiliser is therefore often applied to increase forage production and extend the lactation. Dairy cows generally graze forages on-farm all year round with the exception of cool regions where animals are grazed off during the winter months, and wet areas, where cows can be kept on feeding platforms for several hours a day when soils are wet (de Klein & Ledgard, 2001). Perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) are the most common forages in these dairy systems in New Zealand.

There is current pressure to increase food supply worldwide to feed an ever growing population. This combined with the New Zealand government's target to "increase the ratio of exports to GDP by 40% by 2025" (currently ~30%) (New Zealand Government, 2015) raises concerns regarding the environmental consequences of intensifying the dairy industry. This is especially important, since New Zealand dairy exports are marketed with a 'clean-green' image. New Zealand exports 95% of the milk it produces and although New Zealand is the world's largest exporter of dairy products, it only

produces 3% of the world's milk (DairyNZ, 2016). Dairy exports equated to 22% of New Zealand's total export earnings for the financial year ending March 2015 (New Zealand Government, 2015). Evidence of the intensification of the New Zealand dairy industry is shown by an increase in dairy cow numbers through time (Figure 2.1a). In the 2015-2016 season, New Zealand dairy cow numbers were at 5 million, whereas 20 years ago these were <3 million (Figure 2.1a). The amount of land (effective hectares) used for dairy has also increased, but not at the same rate, meaning that the stocking rate, or number of cows per hectare of land used for dairy is increasing (Figure 2.1a). The average stocking rate currently sits at 2.85 cows ha^{-1}, compared with 2.4 cows ha^{-1} 20 years ago (Figure 2.1a). On a positive note, large increases in milksolids production per cow have been made over the past 20 years, in the 2015-2016 season, mean per cow production was 372 kg MS cow^{-1}, compared with 283 kg MS ha^{-1} 20 years ago (Figure 2.1b).

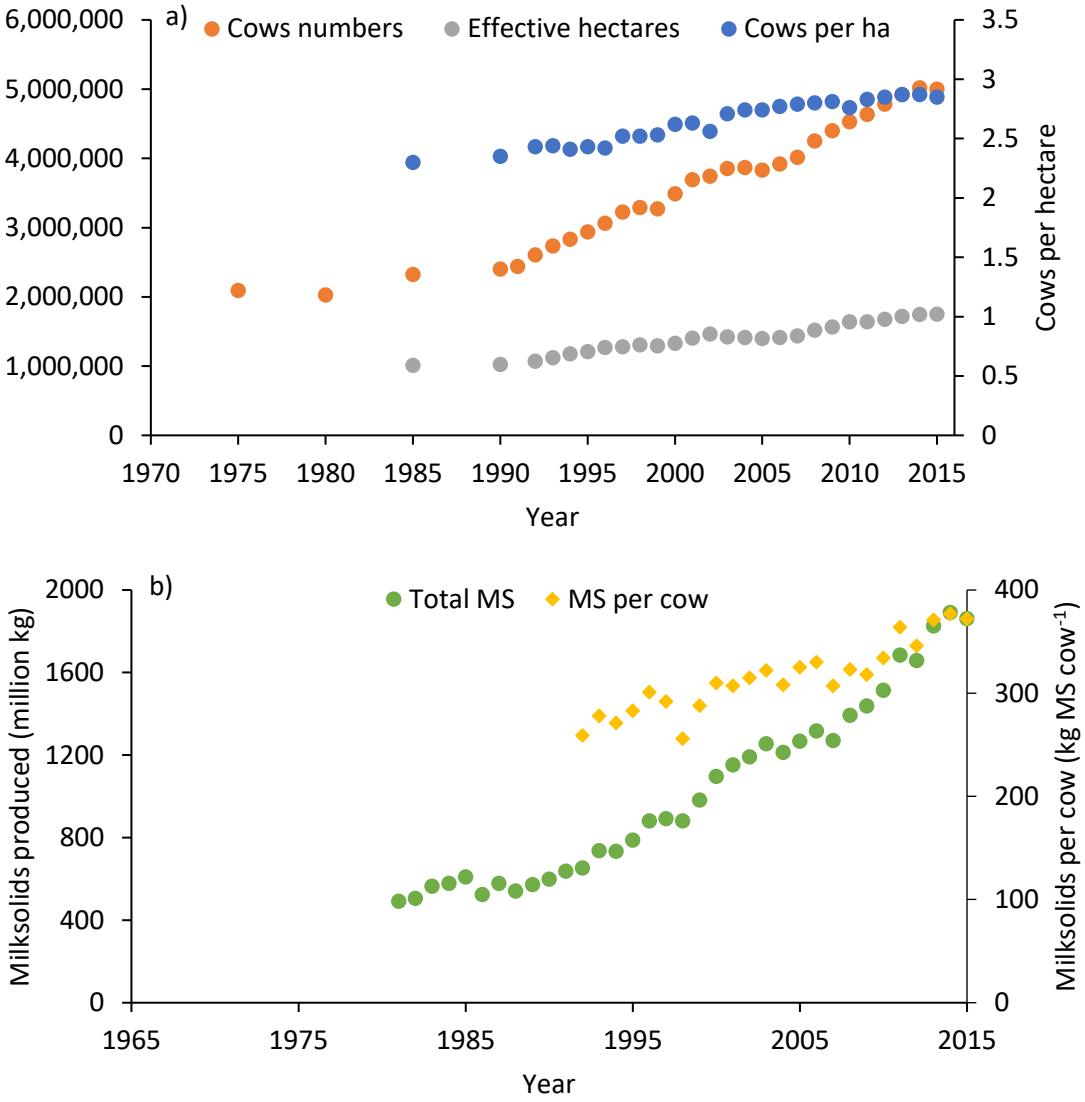


Figure 2.1 New Zealand dairy industry statistics: a) cow numbers, effective hectares in dairy, stocking rate (cows ha^{-1}), and b) milksolids production per cow (kg MS cow^{-1}) (from LIC & DairyNZ, 2016).

In 2014, the New Zealand Government issued a National Policy Statement on Freshwater Management (Ministry for the Environment, 2014). Under this policy, Regional Councils have to ensure that freshwater quality standards are met in rivers and lakes within their region. Thus the current challenge is for the dairy industry to be more profitable while remaining sustainable and reducing the impact on the environment.

2.2.2 Grazed forage systems

In grazed grassland systems, animals only use a small proportion of the N they ingest, and >60% is returned as urine and dung. Over 60% of this is as urine, of which 70% is present in the form of urea (Haynes & Williams, 1993). The N concentration under a cow urine patch can be equivalent to 700-1200 kg N ha^{-1} (Cameron *et al.*, 2013). Each urine patch has an area of around 0.2-0.4 m^2 (Moir *et al.*, 2011) and a cow may urinate 8-12 times a day (Haynes & Williams, 1993). These urine patches can cover 20-30% of a grazed field area per year, depending on the stocking rate (Haynes & Williams, 1993; Moir *et al.*, 2011).

An extensive review by Selbie *et al.* (2015) has provided an update on the current state of knowledge regarding urine patch N dynamics, the implications of urine patches for N cycling and losses, and mitigation strategies. Their *meta*-analysis of published data re-characterised the average dairy cattle (grazing a predominantly pasture diet) urine patch to have an average urine-N concentration of 6.9 g N L^{-1} (n = 51), average volume of 2.1 L (n = 8), deposited onto an average wetted area of 0.24 m^2 (n = 6). Using these characteristics, they calculated an average urine-N loading rate of 613 kg N ha^{-1}, but reported rates for cattle range from 200 to 2000 kg N ha^{-1}. On average urine was described to be deposited 10-12 times per day. Nitrogen and water intake were described as the most important factors influencing the concentration of N in the urine, however, urine-N concentration also varied seasonally, with animal reproduction status and the time of the day. The authors cautioned that *within* species variation in urine-N concentration tends to be greater than that found *between* species. Urine volume, was described to be most influenced by water intake.

2.2.3 The nitrogen cycle in agricultural systems

In soils, N is present in four main forms: 1) organic matter (plant material, fungi and humus); 2) soil organisms and microorganisms; 3) ammonium ions (NH_4^+) held by clay minerals and organic matter; and 4) mineral N in soil solution (including NH_4^+, NO_3^- and low concentrations of nitrite (NO_2^-) (Cameron *et al.*, 2013). The N cycle describes the gains, losses and transformations of N within the soil, plant and atmosphere system (Figure 2.2). Over 95% of the earth's total N is present as organic forms which need to be broken down into plant-available mineral forms (NH_4^+ and NO_3^-) by soil processes (McLaren &

Cameron, 1996). The following N balance equation can be used to describe the amount of mineral-N in the soil at any one time:

$$N = N_p + N_b + N_f + N_u + N_m - N_{pl} - N_g - N_i - N_l - N_e$$

where *p* is precipitation (and dry deposition), *b* is biological fixation, *f* is fertiliser, *u* is urine and dung returned to the soil, *m* is mineralisation, *pl* is plant uptake, *g* is gaseous losses (including ammonia (NH_3), dinitrogen (N_2), nitrous oxide (N_2O) etc.), *i* is immobilisation, *l* is leaching, and *e* is erosion (including surface runoff) (Cameron *et al.*, 2013). It is important that any mitigation options which attempt to reduce N leaching losses do not cause increases in other loss pathways (e.g. losses of the greenhouse gas N_2O by denitrification (Luo *et al.*, 2000)) as this would be "pollution swapping". Management options to reduce N_2O emissions from grazed systems have been thoroughly reviewed by Luo *et al.* (2010) and in an earlier review specific to New Zealand agriculture by de Klein and Ledgard (2005).

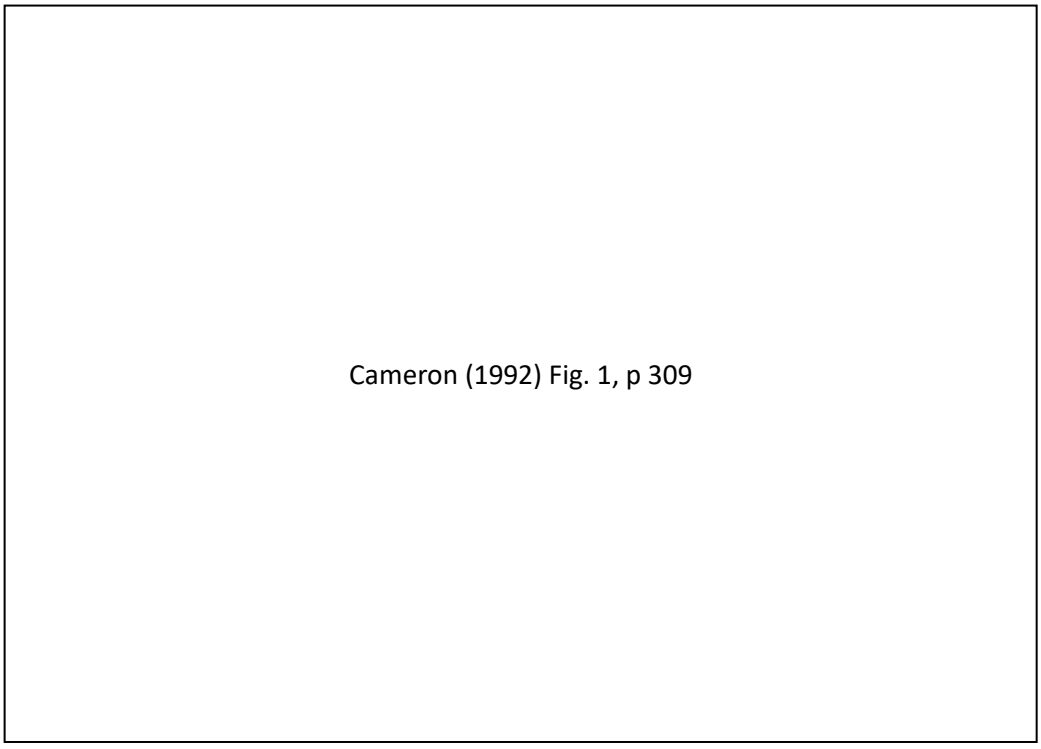


Figure 2.2 The soil/plant nitrogen cycle. From Cameron (1992).

Soil N cycling processes relating to urine deposition

Urea hydrolysis

The first process to occur in soils following the deposition of urine is urea hydrolysis. This involves the rapid hydrolysis of urea to ammonium carbonate. Ammonium carbonate quickly dissociates to NH_4^+ which can be converted to NH_3 gas as illustrated in the equation below (Cameron *et al.*, 2013):

$$(NH_2)_2CO + 2H_2O \rightarrow (NH_4)_2CO_3 \leftrightarrows NH_4^+ + NH_3\uparrow + CO_2 + OH^-$$

Within 48 hours 80% of the urea is hydrolysed (Vallis *et al.*, 1982). The hydroxide (OH^-) ions produced during this process can raise the pH of the soil up to ~pH 8 in the five days following urine deposition. This is subsequently decreased slowly during the nitrification process over a 2-4 week period (Haynes & Williams, 1992).

Nitrification

Nitrification is a soil process which involves the oxidation of NH_4^+ or NH_3 to NO_3^-. This is mainly carried out by autotrophic bacteria in aerobic conditions and occurs in two steps as described by the following reactions:

$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 2H_2O + 4H^+ + \text{energy}$$

$$2NO_2^- + O_2 \rightarrow 2NO_3^- + \text{energy}$$

The first step is ammonia oxidation, where NH_4^+/NH_3 is oxidised to nitrite. This reaction occurs in two parts: the oxidation of NH_3 to hydroxylamine (NH_2OH) which is catalysed by ammonia monooxygenase (AMO), and then oxidation of hydroxylamine to nitrite which is catalysed by hydroxylamine oxidoreductase (HAO) (Kowalchuk & Stephen, 2001). Ammonia oxidation is mainly driven by the activity of the AMO enzyme associated with soil ammonia-oxidising bacteria (AOB) such as *Nitrosospira* and *Nitrosomonas* (He *et al.*, 2007; Cameron *et al.*, 2013). Large populations of ammonia-oxidising archaea (AOA) can also be present in soils (He *et al.*, 2007), however in relatively high N concentration areas of soil (such as in animal urine patches), they have been shown to not be as important as AOB (Di *et al.*, 2009b; Di *et al.*, 2010a; Di *et al.*, 2010b; Di *et al.*, 2010c).

The second step is the oxidation of nitrite to NO_3^- which is driven by the nitrite oxidoreductase enzyme of the nitrite-oxidising bacteria *Nitrobacter* (Wrage *et al.*, 2001). This reaction is very rapid, meaning that nitrite doesn't often accumulate in the soil (Cameron *et al.*, 2013).

Nitrification has been shown to occur within 14-29 days of urine deposition (Williams & Haynes, 1994). Factors affecting nitrification rate include: soil texture, soil structure, temperature, moisture, aeration, pH, electrical conductivity, C:N ratio, cation exchange capacity, and organic matter (Subbarao *et al.*, 2006b).

Nitrate leaching

The majority of soil-N is relatively immobile, with the exception of NO_3^- which has a negative charge and is repelled by cation exchange sites on soil colloids. This means it is easily leached when water drains through soil (McLaren & Cameron, 1996). The amount of drainage which occurs and the NO_3^- concentration of soil solution determine how much NO_3^- is leached from the soil (Cameron *et al.*, 2013). Soil solution NO_3^- concentration is dependent on the amount of N applied to soil, rate of plant uptake, as well as nitrification, denitrification and immobilisation rates (Figure 2.2). Nitrate leaching losses are

usually highest in late-autumn, winter and early spring. This is when cool conditions cause plant uptake of NO_3^- to be low, and rainfall exceeds the demand from evapotranspiration causing drainage to occur (Di *et al.*, 1999; de Klein & Ledgard, 2001; Cameron *et al.*, 2013).

Solute transport

Leaching of NO_3^- from the soil usually occurs through a combination of three transport mechanisms: convection, diffusion and dispersion (Figure 2.3) (Cameron & Haynes, 1986). Further detailed information on solute transport mechanisms relating to NO_3^- are described by Cameron and Haynes (1986), Cameron *et al.* (2013), and Nielsen *et al.* (1982). In brief, convection involves NO_3^- being transported with the mass flow of water through soil during drainage events. The amount of solute transported is affected by soil structure and texture which determine the speed at which the water flows through the soil. Diffusion involves the movement of NO_3^- from an area of high NO_3^- concentration to an area of low NO_3^- concentration. This type of transport depends on the moisture content of the soil and the concentration gradient of the solute (in this case: NO_3^-). The third transport mechanism, dispersion (also called hydrodynamic dispersion) involves the mixing or equalisation of solute distribution which occurs due to the mechanical action of water moving through the soil matrix. The variations in pore size and the tortuosity of soil pores cause a range of different water flow rates and flow path lengths (Cameron *et al.*, 2013). The combined effects of these transport mechanisms have been modelled and are described by the following equation:

$$\frac{\partial c}{\partial t} = E\frac{\partial^2 c}{\partial x^2} - U\frac{\partial c}{\partial x}$$

where *E* is the diffusion coefficient (often called the apparent diffusion) and represents the sum of molecular diffusion plus hydrodynamic dispersion; *c* is the concentration of NO_3^- (μg mL^{-1}); *t* is time (days); *U* is the average pore velocity (cm day^{-1}) (rate of water flow ÷ volumetric water content of the soil); *x* is the linear distance in the direction of the flow (cm) (Cameron & Haynes, 1986). Other mechanisms which can occur under some conditions such as anion exclusion, anion adsorption, and macropore flow are also described by Cameron and Haynes (1986) and are illustrated in Figure 2.3.

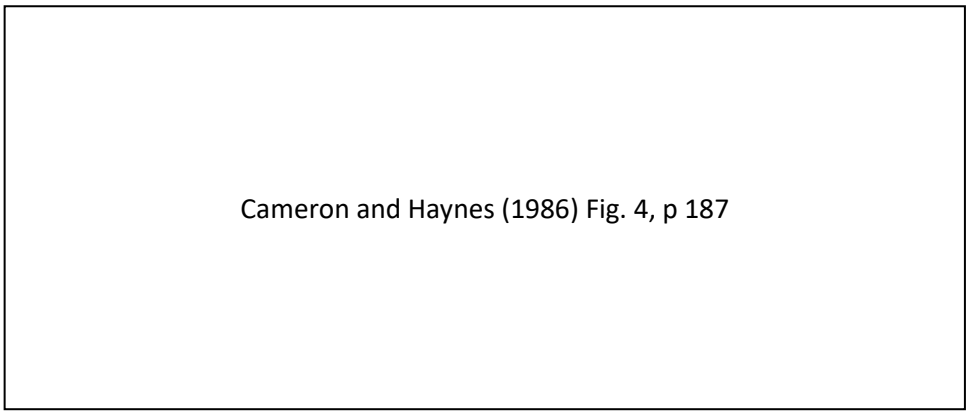


Figure 2.3 Schematic diagram of various components of NO_3^- leaching: a) convective transport alone, b) convection-diffusion-dispersion, c) anion exchange, d) anion adsorption, and e) macropore bypass and macropore leaching. From Cameron and Haynes (1986).

Factors affecting N leaching from urine patches

Nitrate leaching sources, factors and mitigation options have been previously described (Di & Cameron, 2002; Cameron *et al.*, 2013). Factors described included: soil factors such as texture and macropores; climate conditions whereby higher drainage conditions can occur in wetter years (particularly during wet winters), and hot summers can limit urine-N uptake leading to an accumulation of NO_3^- and greater leaching losses in the following winter; and application rates of N sources (urine, fertiliser, effluent etc.) have been shown to significantly increase leaching losses. Similarly, a study by Barraclough *et al.* (1992) showed N leaching losses increased rapidly when annual fertiliser-N inputs were higher than 400 kg N ha^{-1} (Figure 2.4). Processes and factors affecting leaching from urine patches as well as management of leaching from urine have recently been described in a review by Selbie *et al.* (2015).

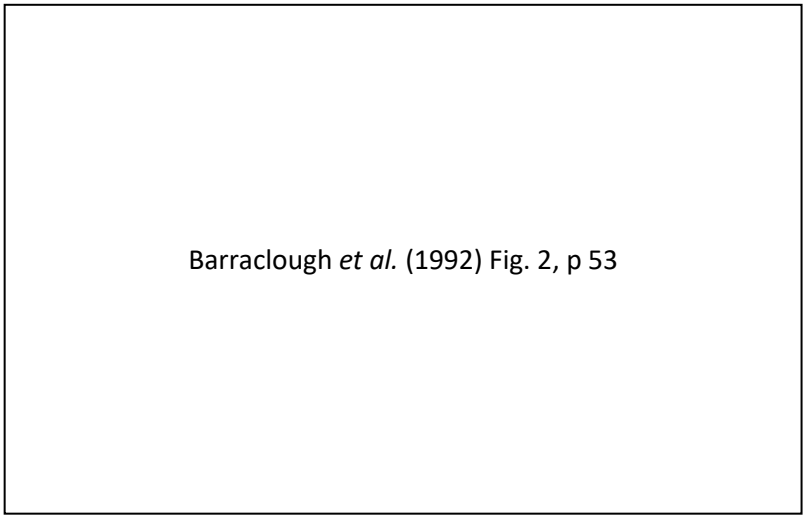


Figure 2.4 Relationship between fertiliser application and annual nitrate leaching losses from grazed and cut plots. From Barraclough *et al.* (1992).

2.2.4 Forage species effects on nitrate leaching

Nitrogen uptake

Nitrogen exists in many forms within plants including protein, peptides, free amino acids, other organic forms, and inorganic forms such as NO_3^- and nitrite. Nitrogen plays a critical role in plant functional enzymes (e.g. rubisco) (Chapman *et al.*, 2014). As previously mentioned NH_4^+ and NO_3^- are the main forms of N taken up by plants (Haynes, 1986b). However, plants can take up some organic forms of N (Nasholm *et al.*, 1998; Hodge *et al.*, 2000; Harrison *et al.*, 2007), and can also absorb N such as NH_3 through their leaves (Haynes, 1986b; Sommer & Jensen, 1991). Nitrate is often the most available form to plants due to the rapid nitrification of NH_4^+ to NO_3^-. Its negative charge also means that it is more mobile in soils. The processes of plant nutrient uptake are described by Whitehead (2000) and uptake of NH_4^+ and NO_3^- by plants are described by Haynes (1986b). Factors affecting plant N uptake include: repression of NO_3^- uptake by NH_4^+, pH in the rhizosphere, interactions among ions, supply of photosynthates (e.g. carbohydrates), temperature, and mycorrhizal associations (Haynes, 1986b). Plant transpiration demand pulls NO_3^- towards the plant roots by mass flow, and this is the main way in which NO_3^- moves towards plant roots (Crush *et al.*, 2007). Crush *et al.* (2005) showed a strong positive correlation between plant dry weight and the proportion of a pulse of labelled NO_3^- which was intercepted.

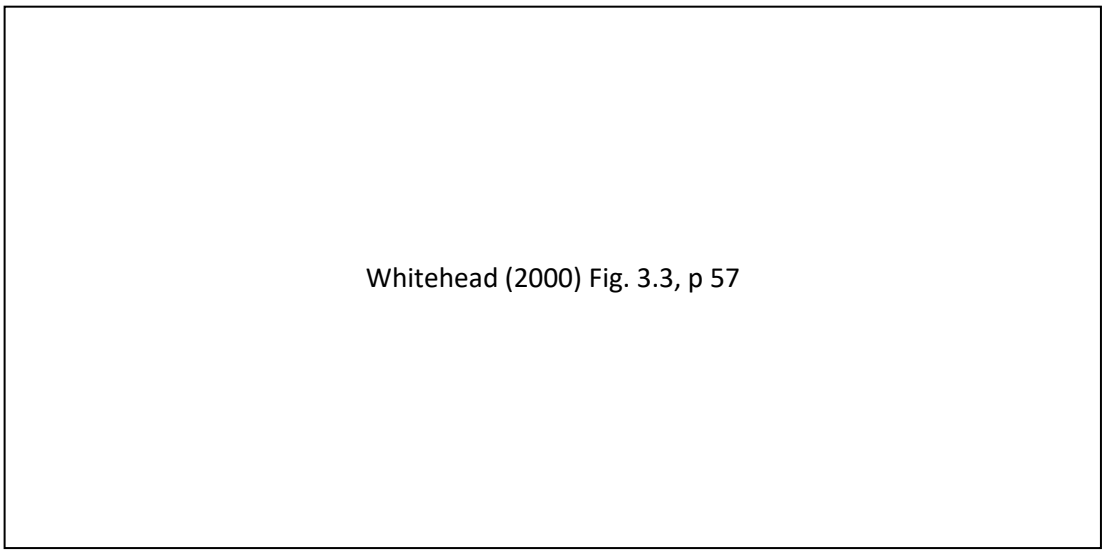


Figure 2.5 General relationship between growth or yield and concentration of a nutrient element in the plant tissue. From Whitehead (2000).

In a review of plants for dairy grazing systems where N leaching losses are limited, Chapman *et al.* (2014) describe the critical internal requirement for N (N_{CInt}), and the critical external requirement for N (N_{CExt}) as being the two parameters which can be used to characterise the N nutritional physiology of plants. These requirements are determined relative to some percentage of maximum biomass yield, N_{cInt} is the N% required in dry matter (DM) to reach this percentage of biomass yield (an example is

given in Figure 2.5), and N_{CExt} is the N supply to the soil required to reach this percentage of biomass yield. Critical internal and external requirements for N differ among plant species. Chapman *et al.* (2014) highlight how knowledge of N_{cInt} and N_{CExt} could help to reduce the N surplus in grazing animals and thereby reduce urine-N excretion. They reviewed the current literature on the use of alternative species to manage NO_3^- leaching and outlined a major knowledge gap: "there is insufficient information for alternative pasture species from which robust analyses of the relationship between feed supply and N balance in pastures can be built. This is a major shortcoming in the knowledge base required to develop more sustainable, pasture-based livestock production systems that meet future requirements for environmental quality" (Chapman *et al.*, 2014). Similarly, in a recent review of simple and diverse (three or more sown species) pastures that address the soil-plant-animal relationships with N leaching, diverse pastures had greater herbage yields (Vibart *et al.*, 2016). In general, increasing diversity was shown to increase sward N uptake through the variation in root depth and seasonal plant growth of the different species in the diverse mixtures complementing each other. However, they also found that the presence of certain species was more relevant to herbage yield and N dynamics than the number of species in a mixture. Studies covered in their review included a wide range of grasses, legumes, and herb species as part of simple or diverse mixtures (Vibart *et al.*, 2016).

In a study with perennial ryegrass, Crush *et al.* (2007) reported that the rate of plant growth and size of the root system were important regulators of NO_3^- interception, but that root depth was of secondary importance. Similarly, Malcolm *et al.* (2014) found that NO_3^- leaching was linked to winter daily N uptake of pasture where a 1 kg N ha^{-1} increase in average daily N uptake was shown to reduce total NO_3^--N leached by 132 kg N ha^{-1} during the 10-month measurement period. Further research is needed to quantify the effects of higher winter growth or larger root systems on N leaching, and could confirm whether higher N uptake occurs compared with typical perennial ryegrass-white clover, particularly in high risk urine patch areas. This knowledge is required to assess whether these species could be used to reduce NO_3^- leaching losses. The current review will focus on the following alternative forages: Italian ryegrass, lucerne, and plantain to potentially reduce N leaching from grazed systems.

Italian ryegrass

Italian ryegrass (*Lolium multiflorum* Lam.) has larger leaves and tillers, with less prolific tillering than perennial ryegrass. Its growth tends to be erect, and though not a perennial species, Italian ryegrass can produce high yields of quality forage for up to 3 years. Growth during winter and early spring tends to be greater than perennial ryegrass, however, it is often poorer in summer and autumn (Kemp *et al.*, 1999; Stewart & Charlton, 2006).

A limited number of studies have measured NO_3^- leaching losses from Italian ryegrass, and recently Malcolm *et al.* (2014) reported NO_3^--N leaching losses 24-54% lower beneath Italian ryegrass-white

clover, compared to other forage species in their experiment. Similarly, DM yield was 11-58% higher in the season following establishment. The authors attributed the observed reduction in leaching to higher plant winter activity. Then, in a more detailed study comparing Italian ryegrass and tall fescue (*Festuca arundinacea* Schreb.), Malcolm *et al.* (2015) found that plant growth rate was more important than root architecture (root: length, surface area, length density, and uptake efficiency) for uptake of N from soil during the winter. In a glasshouse study, Moir *et al.* (2013) showed Italian ryegrass had the highest DM yields and the lowest leaching loss following a 700 kg N ha^{-1} urine application (134 and 130 kg N ha^{-1} for 'Feast 2' and 'Tama', respectively). Strong negative relationships between N leaching loss, plant N uptake and root mass were shown. Similarly, Nichols and Crush (2007) showed drainage volumes and NO_3^- contents of leachate from hybrid/Italian ryegrass cultivars were significantly lower, and absorption of ^{15}N higher, compared with perennial ryegrass. This was reinforced by Popay and Crush (2010) who showed 95% lower NO_3^- leaching losses from Italian ryegrass than perennial ryegrass following application of synthetic cow urine (600 kg N ha^{-1}). Recovery of ^{15}N was also lower for perennial ryegrass, which was attributed to its lower plant and root mass. In an earlier study, Italian ryegrass was shown to have the second highest ^{15}N recovery (0.34 mg g^{-1} root) of the species tested, and observations indicated that Italian ryegrass has the ability to grow roots deeper than 1 m (Figure 2.6) (Crush *et al.*, 2005).

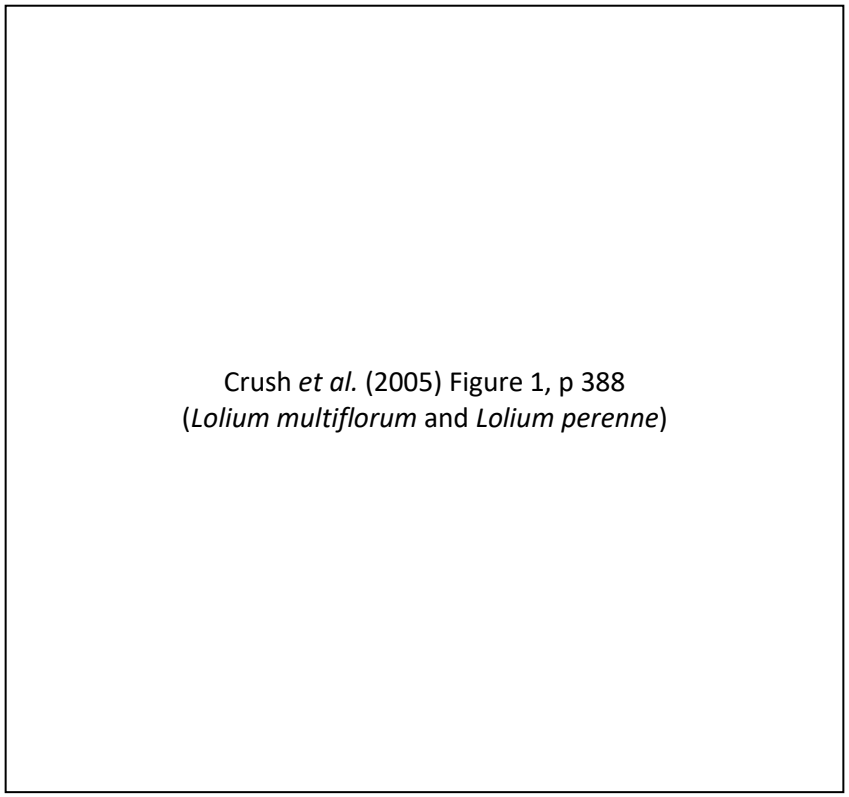


Figure 2.6 The proportion of total root mass recovered in 0.1 m depth increments to 1 m of tubes containing sand. From Crush *et al.* (2005).

Under rain-fed conditions, no NO_3^- leaching was observed as measured by a water flux meter beneath Italian ryegrass cut and carry crops, despite application of N in both pig slurry and fertiliser forms (250-272 kg N ha^{-1}) (Domingo *et al.*, 2007). Similarly, Judge *et al.* (2003) showed Italian ryegrass following a maize crop removed 55-60% of applied N, and NO_3^- leaching losses ranged from 17-34 kg N ha^{-1}. A linear yield response to N fertiliser from 40-160 kg N ha^{-1} was observed without increased N leaching (Judge *et al.*, 2003). Italian ryegrass has been described as a species which could play a role in reducing forage N leaching losses (Nichols & Crush, 2007; Moir *et al.*, 2013), while traditionally sown perennial ryegrass may be less suitable (Moir *et al.*, 2013).

In contrast to this, Aavola and Karner (2008) noted that perennial ryegrass cultivars were more efficient than Italian ryegrass in taking up N from the soil and fertiliser during simulated grazing, although in N deficient soils, Italian ryegrass had the best N uptake. Similarly, in a mini-rhizotron study (2.4 m deep), Kristensen and Thorup-Kristensen (2004) measured Italian ryegrass roots to only 0.6 m. Nitrogen uptake was 127.4 kg N ha^{-1}, however, residual soil NO_3^- remained reasonably high at 87 kg N ha^{-1} at the end of the experiment. In another study, NO_3^- leaching measured by porous ceramic suction cups at 0.6 and 1.5 m depths was <1 kg N t^{-1} DM produced for barley (*Hordeum vulgare* L.)-oats (*Avena sativa* L.)-Italian ryegrass cropping sequence. Although when urine was applied (800 kg N ha^{-1}), NO_3^- leaching losses were almost 2 times greater from beneath Italian ryegrass than the oats (369 vs 134 kg N ha^{-1}) (Beare *et al.*, 2010).

One possible mechanism by which Italian ryegrass could potentially reduce N leaching losses could be through biological nitrification inhibition (BNI). Italian ryegrass 'Nioudaichi' (*Lolium perenne* L. ssp. Multiflorum (Lam.) Husnot) has been shown to have some BNI activity: total BNI released from four plants was 13.5 AT units, and specific BNI was 2.6 AT units g^{-1} root dry weight (Subbarao *et al.*, 2007). One AT unit is defined as equal to the inhibitory effect of 0.22 µM of allylthiourea (a standard inhibitor) in an assay containing 18.9 mM of NH_4^+ (Subbarao *et al.*, 2006a).

Lucerne

Lucerne (*Medicago sativa* L.), also known as alfalfa, is a perennial legume which produces erect stems from a crown. It is commonly grown in dryland areas of New Zealand as its taproot allows the plant to extract water from deep in the soil, and therefore it shows a greater tolerance to drought than most other forage species. Lucerne can produce over 20 t DM ha^{-1} on well drained, high fertility soils (pH >5.8) (Vartha, 1973; Thomson, 1977; Kemp *et al.*, 1999; Brown *et al.*, 2000; Brown *et al.*, 2003). The on-farm use of lucerne for grazing and conserved feed has increased in New Zealand in recent years with new cultivars coming onto the market, including more winter-active ones (Harvey *et al.*, 2014). In general, the more winter-dormant material has been considered more suitable for South Island farming systems (Harvey *et al.*, 2014).

A number of studies have investigated the ability of lucerne to extract water from the soil (Ayars *et al.*, 2009; Murray-Cawte, 2013; Sim, 2014) and lucerne was shown to extract water from a depth of at least 2.3 m (maximum depth measured) (Voorhees & Holt, 1969; Brown *et al.*, 2005; Moot *et al.*, 2008) compared with 1.5 m for perennial ryegrass (Figure 2.7) (Moot *et al.*, 2008). Hobbs (1953) showed lucerne utilised subsoil moisture reserves to a depth of at least 5.5 m.

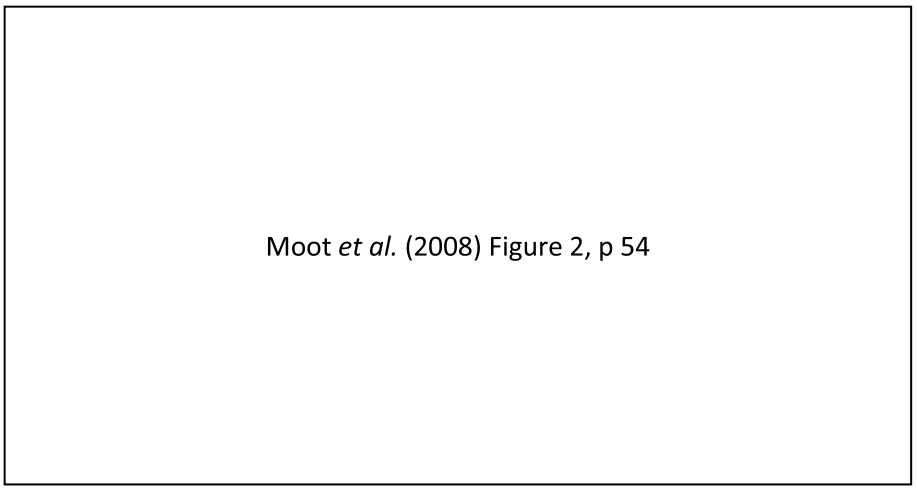


Figure 2.7 Water extraction (mm) from each 0.1 m soil layer from 0-2.3 m depth for lucerne (circles) and grass based pasture (triangles) on a deep Wakanui silt loam (solid symbols) or a Lismore (A) very stony loam and Lismore (B) stony loam (open symbols). From Moot *et al.* (2008).

Water use efficiencies from 14.1 to 40 kg DM ha^{-1} mm^{-1} have been reported for lucerne (McKenzie *et al.*, 1990; Paul, 1991; Moot *et al.*, 2008; Tonmukayakul *et al.*, 2009; Kearney *et al.*, 2010; Moot, 2012).

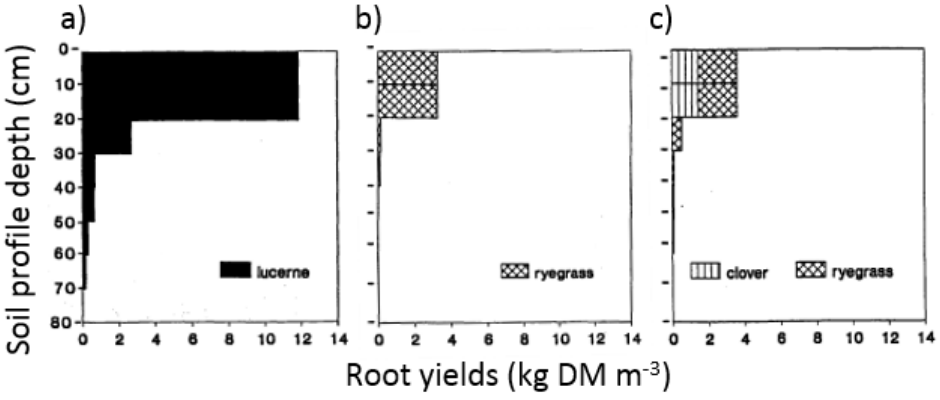


Figure 2.8 Root dry matter yields of: a) lucerne, b) perennial ryegrass, and c) perennial ryegrass-white clover at different depths of the soil profile. Adapted from Gyamtsho (1990).

Lucerne is known for its deep rooting capacity, and a number of studies have reported lucerne's root depth and root mass. Lucerne roots have been reported beyond 6 m (Mathers *et al.*, 1975) and even down to 10 m for mature plants in favourable soil (Forde *et al.*, 1989). The mass of lucerne roots

measured at 0.1 m increments to a depth of 0.8 m is described in Figure 2.8 (Gyamtsho, 1990; McKenzie *et al.*, 1990). Similarly, Eberbach *et al.* (2013) investigated the growth and development of lucerne roots using rhizolysimetry to a depth of 2.05 m.

The ability of lucerne to take up N has also been reported in a number of studies. Accumulated N yields of >500-834 kg N ha^{-1} have been shown (Boawn *et al.*, 1963; Mills & Moot, 2010; Black & Moot, 2013). Lucerne has been shown to extract NO_3^- from the soil down to 1.2 m (Sainju & Lenssen, 2011) and some below 1.2 m (Huang *et al.*, 1996). Similarly, an early study by Stewart *et al.* (1968) reported that lucerne might remove NO_3^- from the groundwater, where the water table was within 6.1 to 7.6 m of the surface. Lucerne has been shown to be an efficient scavenger of mineral N (Frankow-Lindberg & Dahlin, 2013). The relationship between lucerne N uptake, and both shoot yield and LAI has recently been described as linear (Sim, 2014).

A limited number of studies have investigated the effect of lucerne on NO_3^- leaching. A recent New Zealand study by Betteridge *et al.* (2007) measured NO_3^- leaching from a lucerne crop using ceramic suction cups (0.6 m deep). Lucerne leached 10-24 kg NO_3^--N ha^{-1} y^{-1}, the same or slightly more NO_3^--N per hectare than pasture. In this study, both the lucerne and pasture were cut and carry systems, whereas grazed systems where urine and dung are returned would be expected to have higher losses of NO_3^-. Some studies have examined NO_3^- leaching from lucerne applied with animal manure. Dairy manure applied to lucerne at a low rate of 112 kg N ha^{-1} was shown to have no adverse effect on herbage production or NO_3^- in soil water measured from porous ceramic cup samplers at 0.3, 0.6, 0.9 and 1.2 m depths (Daliparthy *et al.*, 1994). On land treated for 3 years with differing quantities of manure, lucerne was shown to remove water and NO_3^--N to 1.8 m in the first year and to 3.6 m in the second year. Yield, NO_3^--N and P contents of lucerne were increased by manure and total N uptake was directly related to yield (Mathers *et al.*, 1975).

Some studies have compared the effectiveness of N_2-fixing vs non-fixing lucerne to take up N. Russelle *et al.* (2007) showed N_2-fixing lucerne removed nearly 60% more soil and manure N on an abandoned dairy feedlot than non-fixing lucerne, though neither prevented ground water contamination by NO_3^-. However, earlier studies suggested non-fixing lucerne to be more effective (Blumenthal & Russelle, 1996; Lamb *et al.*, 2005) and some showed that both would be effective (Russelle *et al.*, 2001). In another abandoned feedlot study, N uptake was 2.5-3 times greater for lucerne than for corn, soil NO_3^- levels were lower, and NO_3^- remained closer to the soil surface (Schuman & Elliot, 1978).

Other studies which have measured NO_3^- in leachate beneath lucerne include Fox *et al.* (2001) who measured concentrations of ~4 mg NO_3^--N L^{-1} in leachate below lucerne using pan lysimeters (1.2 m

deep). Similarly, Bergstrom (1987) measured NO_3^--N in leachate of 0-8.2 kg N ha^{-1} yr^{-1} from a lucerne ley using two different methods: tile-drained large-plots (0.36 ha, 1 m deep), and disturbed large lysimeters (27 m^2, 1 m deep). Owens (1990) reported NO_3^--N concentrations of <5 mg L^{-1} in leachates from monolith lysimeters (8.1 m^2, 2.4 m deep) planted with lucerne-grass mixtures. Where lucerne was incorporated into a riparian buffer, NO_3^--N concentrations from lysimeters were considerably less than under the adjacent crop (Yamada *et al.*, 2007). In glasshouse soil columns planted with lucerne, Chavez *et al.* (2012) compared NO_3^- concentration in leachate following treatment with different waste water treatments. Concentrations of 203.7-305.9 mg NO_3^- L^{-1} were shown.

A limited number of studies have examined NO_3^- leaching from lucerne, but no studies were found which measured NO_3^- leaching from grazed lucerne in dairy systems. There is a specific gap in our knowledge about the amount of NO_3^- leaching loss that occurs from urine patches on lucerne forage. It is important to be able to quantify these losses, in order to gain a better understanding of how N moves through the soil under these systems.

Plantain

Plantain (*Plantago lanceolata* L.) is a narrow-leaf perennial herb, which belongs to the Plantaginaceae family (Al-Mamun *et al.*, 2008a). Plantain is used in herbal medicine (Gálvez *et al.*, 2005) and is broadly distributed in temperate grasslands worldwide (Stewart, 1996). The suitability of plantain as a pasture species was reviewed by Stewart (1996). The review describes the two main cultivars available at the time: 'Ceres Tonic' and 'Grasslands Lancelot', as well as plantain establishment, soil fertility requirements, drought tolerance, pests and diseases, herbage productivity, palatability to grazing animals, animal performance, mineral levels, nutritional value, and its unique chemical properties (Stewart, 1996). Plantain has been described as a rapidly establishing, drought tolerant forage which has the ability to grow on a wide range of agricultural soils, its leaves are highly palatable to grazing animals (Stewart, 1996), and provide a mineral rich forage (Pirhofer-Walzl *et al.*, 2011). Its deeper roots mean it can have a competitive advantage over shallower rooted grasses for water and nutrients (Stewart, 1996). Plantain is also tolerant of many common diseases and pests (Marak *et al.*, 2000, 2002; Biere *et al.*, 2004).

Many studies have shown that when plantain is incorporated into a forage mixture, it can produce similar or greater DM yields (Malcolm, 2013; Nobilly *et al.*, 2013; Totty *et al.*, 2013; Woodward *et al.*, 2013; Macfarlane *et al.*, 2014), and milk production (Minnee *et al.*, 2012; Woodward *et al.*, 2012; Totty *et al.*, 2013; Woodward *et al.*, 2013; Edwards *et al.*, 2015; Box *et al.*, 2016) to perennial ryegrass-white clover.

The inclusion of plantain in mixed swards has been shown to reduce the amount of N excreted in urine, while maintaining similar herbage yields to standard perennial ryegrass-white clover (Woodward *et al.*, 2012; Totty *et al.*, 2013; Edwards *et al.*, 2015). Urinary-N concentrations of 0.26% and 0.34% have been reported for mixtures containing plantain, compared with 0.62% and 0.57% for perennial ryegrass-white clover (Woodward *et al.*, 2012; Totty *et al.*, 2013). Similarly, in a recent study, Box *et al.* (2016) measured urine-N excretion from a perennial ryegrass-white clover, a 50:50 plantain:perennial ryegrass-white clover, and a 100% plantain treatment in autumn, and reported concentrations of 5.4 g N L^{-1}, 3.6 g N L^{-1}, and 2.4 g N L^{-1}, respectively. Lower NH_3, urea, and creatinine levels in the urine of cows grazing plantain were also reported. The authors suggested one explanation for this lower N excretion could be due to differences in urine volume, of which there was some evidence shown in the reduced urine creatinine levels for cows grazing plantain forages. They said this may have been caused by plant secondary metabolites or increased water intake due to the lower DM% of plantain observed in their study (Box *et al.*, 2016). The 'Grasslands Lancelot' cultivar of plantain, has previously been described as having both an antibiotic effect on rumen flora and a diuretic effect (Rumball *et al.*, 1997). Similarly, in a recent study using the 'Ceres Tonic' cultivar of plantain, aucubin and acteoside were shown to reduce NH_3 production in the rumen *in vitro* (Navarrete *et al.*, 2016). Aucbin by inhibiting rumen fermentation, and acteoside by increasing gas production and possibly by being used as an energy source for microbial growth (Navarrete *et al.*, 2016). Both have the potential to reduce the N losses in the urine of ruminant animals (Navarrete *et al.*, 2016). In a modelling study, where 20% and 50% diverse mixtures containing plantain and other species were incorporated into a whole farm system, a 3.3-8.1% reduction in urinary-N excretion was predicted on an annual basis, when compared with a standard perennial ryegrass-white clover system (Khaembah *et al.*, 2014). Al-Mamun *et al.* (2008a) showed 23-33% lower N in urine from sheep fed a diet containing 10% plantain, compared with 100% hay diet (containing orchardgrass, *Dactylis glomerata* L., and reed canarygrass, *Phalaris arundinacea* L.).

In a study with heifers, spring urine-N concentrations and urine-N excretion were shown to be lower for heifers grazing plantain (2.9 g kg^{-1} and 87 g day^{-1} $heifer^{-1}$, respectively), compared with perennial ryegrass-white clover (4.8 g kg^{-1} and 116 g day^{-1} $heifer^{-1}$, respectively) (Cheng *et al.*, 2017). The authors suggested that the reduced urine-N may have reflected the lower N intake of the heifers (224 vs 348 g day^{-1} $heifer^{-1}$ for plantain and perennial ryegrass-white clover, respectively) but also said that higher water intake due to lower DM of the plantain may have led to increased urine volume, with diluted N concentration (Cheng *et al.*, 2017). In contrast, no significant difference in autumn urine-N concentration between heifers fed a standard perennial ryegrass-white clover diet, compared with one containing plantain was shown by Carr (2015) and Cheng *et al.* (2017).

This reduction in urine-N concentration described in many of the studies above, and thus lower urine-N loading of the urine patch could be one strategy by which plantain could be used to reduce the amount of urine-N which is leached. Many studies describe the potential for reduced N leaching from plantain through this mechanism (Woodward *et al.*, 2013; Khaembah *et al.*, 2014; Edwards *et al.*, 2015; Box *et al.*, 2016; Vibart *et al.*, 2016), however there are very few studies which have measured leaching from a plantain forage. Malcolm *et al.* (2014) measured N leaching losses from a diverse mixture containing plantain and found that N leaching losses were not significantly different from perennial ryegrass-white clover. However, the urine treatment applied did not take account of any reduction in urine-N concentration caused by the presence of plantain, instead standardised urine was used at a rate of 1000 kg N ha^{-1}. With increasing evidence of the presence of plantain to reduce urine-N excretion by grazing animals, there is a need for more information on N leaching losses from these forages which take into account the effect of the urine-N excretion of animals grazing these forages.

2.2.5 Gibberellic acid

Gibberellic acid (GA), also known as Gibberellin A3, is a hormone which occurs naturally in most plants. First identified in Japan in 1935, it is a simple gibberellin that is responsible for increased stem elongation and leaf expansion (Matthew *et al.*, 2009). A review paper by Matthew *et al.* (2009) outlined the history of gibberellic acid discovery, isolation and historical use. They summarised pasture responses to GA, GA application rates, and the main findings of a selection of experiments. In general, response per gram of GA decreased with increasing rate of GA applied, ≤10 g GA ha^{-1} was suggested to be economically viable. Recent studies in Canterbury, New Zealand, have shown a single application of GA to a perennial ryegrass-white clover pasture increased DM production compared to a no GA control by 39% (24 g GA ha^{-1}) (van Rossum *et al.*, 2013) and 18-51% (8 g GA ha^{-1}) (Jiang *et al.*, 2011) after the first harvest. Similarly, Bryant (2012) showed that a single application of GA in mid-August improved DM yield by 59-96% compared to the control, in 3 out of 4 years, from 2009-2012. Herbage DM yield increases of 45-74% (GA only) and 26-36% (GA + N) were shown for perennial ryegrass-white clover applied with GA (8 g GA ha^{-1}), compared with the no GA control, and the urea-only (50 kg N ha^{-1}) treatments, respectively (Bryant *et al.*, 2016). Across a range of New Zealand sites Zaman *et al.* (2014) consistently measured increases in herbage DM yield following an application of GA (20 g GA ha^{-1}). Increases of 13-107% (0.1-1.2 t DM ha^{-1}) were shown for perennial ryegrass-white clover, compared with the no GA control (Zaman *et al.*, 2014). Similarly, when applied with N in the form of 20-40 kg N ha^{-1} urea, additive DM responses were shown for most locations (Zaman *et al.*, 2014). Studies which examine GA application to lucerne are limited, and though yield increases with GA application have been observed (Finn & Nielsen, 1959; Bidlack & Buxton, 1995), decreased root yield was also shown

(Finn & Nielsen, 1959). In contrast, more recent studies have shown that lucerne did not respond to GA application (Carrer *et al.*, 2003; Terzi & Kocacaliskan, 2010).

Ball *et al.* (2012) examined the response of perennial ryegrass in mid-winter and mid-summer to GA (8 g GA ha^{-1}). They found that responses were significant but far smaller in the summer-derived than the winter-derived plants. In a similar experiment, Parsons *et al.* (2013) found a major increase in DM production in winter-derived plants took place at both low and high N, with no evidence of a reduction of N content of tissues. This suggested that extra growth increased N uptake from the soil environment. Their findings supported the hypothesis that there is an element of internal control in how plants respond to 'signals' in their environment, which might be manipulated. They suggested that this offers prospects for reducing environmental impacts (leaching, N_2O) compared with obtaining the same yield increase by adding fertiliser-N. Similarly, Morgan and Mees (1958) described increased uptake of N with GA at the first harvest, though decreases of crude protein (CP) at harvest 2 were often observed. Some studies have also noted increases in total N or CP yield due to DM increases with GA (Morgan & Mees, 1958; Finn & Nielsen, 1959; Biddiscombe *et al.*, 1962). In their recent study, van Rossum *et al.* (2013) found that CP content was decreased by GA application in all pastures except perennial ryegrass-white clover. Other reductions in N and CP content have been reported for forage species (Scurfield, 1958; McGrath & Murphy, 1976; Percival, 1980; Ghani *et al.*, 2014; Zaman *et al.*, 2014; Bryant *et al.*, 2016) and Champeroux (1962) showed GA improved N utilisation through increased DM yield but decreased N uptake and N content. In their first experiment Biddiscombe *et al.* (1962) found no significant difference in total yield of N over all five harvests. Increases in the proportion of clover in perennial ryegrass-white clover has also been shown following GA application (van Rossum *et al.*, 2013; Bryant *et al.*, 2016).

Some side effects of GA application described by Matthew *et al.* (2009) include: later yield depression; an increased shoot:root ratio; decreased tillering and groundcover; stimulation of flowering (some species); forage quality (reduction in CP often reported); and chlorophyll reduction. Gibberellic acid application is frequently followed by a yellowing of foliage, though this effect is less pronounced where N-fertiliser is used together with the GA. Despite reductions in chlorophyll content (Williams & Arnold, 1964; Dijkstra *et al.*, 1990) and temporary chlorosis (Morgan & Mees, 1956; Finn & Nielsen, 1959) being reported with GA application to some forage species, increases in Rubisco activity and leaf photosynthesis have also been described (Treharne & Stoddart, 1968).

There have been many studies carried out using other plant species, which could still have some relevance to pastoral plants. Gibberellic acid application was shown to increase N in *Calendula*

officinalis L. (Mohamed & Ebtsam, 2013), linseed (Khan *et al.*, 2010), and in wheat (Brian *et al.*, 1954). A study by Livné and Vaadia (1965) showed transpiration rate increased following GA application.

Whitehead and Edwards (2015) assessed the potential of gibberellins to reduce N_2O emissions from grazed grassland and concluded that gibberellins could be used to reduce the use of N fertilisers while leading to similar or greater increases in dry matter. They suggested that this could reduce the N intake by ruminants, resulting in lower N excretion. Their modelling estimated reductions in N_2O emissions of 1.6% and 1.3%, relative to an untreated control, for one application of GA in late summer and early spring, respectively. They suggested this could be as high as 5% and 6% when one split application of fertiliser-N is substituted with GA. It is possible that a reduction in excreted N caused by GA application could not only reduce N_2O emissions but could also reduce NO_3^- leaching. They define three key considerations of effects of gibberellins on forage composition: the effect of GA on herbage N concentration, the effect of GA on WSC of the herbage, and the effect of GA on ME. In another modelling study, Ghani *et al.* (2014) predicted reductions in N leaching from 4% to 29% in the scenarios they modelled.

Despite some studies which have examined the effect of GA on N content, chlorophyll content, DM production and many other morphological characteristics of forages, there appears to be no studies which have examined the effect that GA application to forages might have on subsequent NO_3^- leaching. This knowledge gap presents an opportunity to extend our understanding of the effect of GA on the plant-soil system, and determine whether GA has any further environmental benefits to offer. Current literature appears to show conflicting information regarding the effect of GA on plant N and chlorophyll content. Further studies could help to clarify these responses for forage species, and combined with a leaching study, could result in a better understanding of how GA affects the movement of N through the plant-soil system.

2.3 Fate of N in grazed forage systems

A recent review of the fate of urine in grazed forage systems estimated typical values of urine-N recovery to be: 41% plant uptake, 20% NO_3^- leaching, 26% immobilisation (soil), 2% N_2O emissions, and 13% NH_3 volatilisation (Selbie *et al.*, 2015). However, this review did not specifically examine studies using ^{15}N. Using ^{15}N to trace the fate of urine-N provides robust data which are important to improve understanding of the N cycling and losses of a particular system. Many ^{15}N balance studies have traced the fate of urine within forage systems (Table 2.1). These show recoveries of urine-^{15}N ranging from 0.3 to 69.5% for plant shoots, and 0.02 to 19.2% for roots, 12.7 to 63.7% for soil, 0.1 to 62% for N leaching, 0.015 to 2.2% for N_2O emissions, and 0.7 to 50% for NH_3 volatilisation. Only two studies reported separate N_2 emissions (Clough *et al.*, 2001; Selbie, 2014). Selbie (2014) reported a N_2 loss of

26.06% and suggested that this could explain the unaccounted for N in many ^{15}N balance studies. Other previous studies did not measure N_2 or instead combined N_2O and N_2 losses. Variability in urine-^{15}N balance results (Table 2.1) is likely to be from varying rates and times of urine application, varying soil conditions (soil type, fertility, structure, moisture content), study duration and climates. Previous ^{15}N labelled studies have been predominantly performed on either perennial ryegrass (*Lolium perenne*) monocultures or perennial ryegrass and white clover (*Trifolium repens*) mixtures. Two studies traced urine-^{15}N applied to Italian ryegrass (*Lolium multiflorum*), but neither produced a full ^{15}N balance: Sorensen and Jensen (1996) did not measure leaching or denitrification losses, while Malcolm *et al.* (2015) did not measure denitrification losses or ^{15}N in soil. No studies have traced the fate of urine-^{15}N in lucerne forage.

Recovery of ^{15}N in leachate (%) vs ^{15}N in herbage (%), from the studies summarised in Table 2.1, are plotted in Figure 2.9. At a higher recovery of ^{15}N in herbage, the maximum recovery of ^{15}N in leachate reported was lower. Other studies have also observed strong inverse relationships between leachate-N and herbage N uptake (Malcolm *et al.*, 2014; Selbie, 2014).

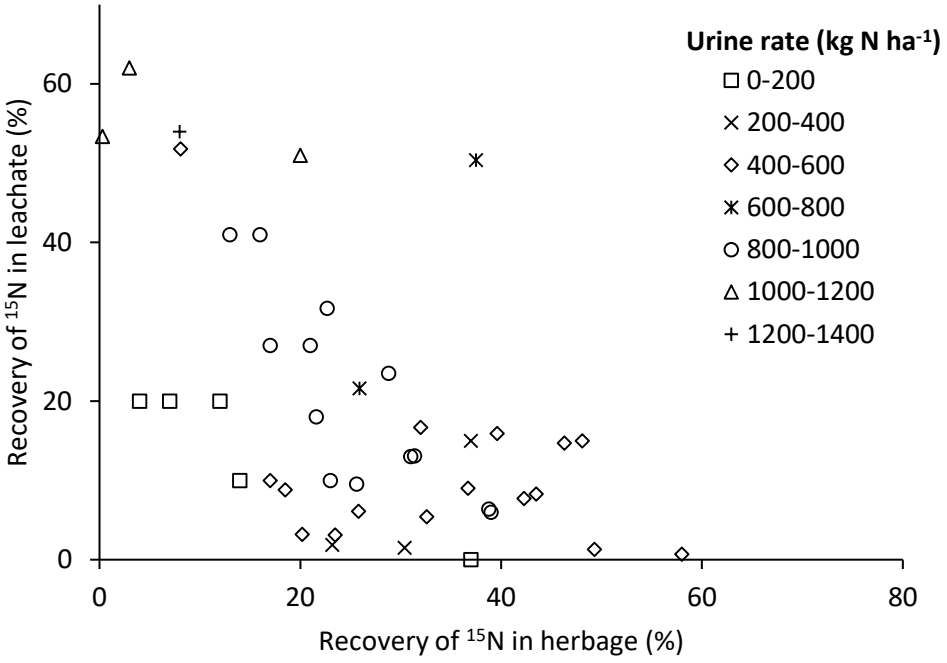


Figure 2.9 Relationship between recovery of ^{15}N in the herbage (%) and ^{15}N in the leachate (%) from studies compiled in Table 2.1.

Table 2.1 A summary of reported ^{15}N balances for urine patches in grazed forage-based systems in the literature.

			% of applied N recovered									
Study	**Urine rate (kg N ha^{-1})**	**Plant type(s)**	**Leaching**	**Forage**		**Soil**	**Denitrification**		**NH_3**	**Days**	**Soil texture**	**Country**
				Shoots	**Roots**		**N_2O**	**N_2**				
Wells *et al.* (2015)	600 winter	*Lolium perenne* L.	-[1]	-	-	-	-	-	25	17	Silt loam	New Zealand
Malcolm *et al.* (2015)	300 autumn	*Lolium multiflorum* L. *Festuca arundinacea* Schreb.	1.52 1.88	30.4 23.2	-	-	-	-	-	150	Fine sandy loam	New Zealand
Selbie (2014)	1000 winter	*Lolium perenne* L.	9.55	25.63		23.3	0.48	26.06	-	368	Sandy loam	Ireland
Buckthought (2013)	800 autumn	*Lolium perenne* L. + *Trifolium repens* L.	50.4	37.5	0.02	-	0.57	-	-	481	Silt loam	New Zealand
Welten *et al.* (2013)	600 late autumn	*Lolium perenne* L. + *Trifolium repens* L.	21.6	25.9	0.03	24.1	-	-	-	300	Sandy loam	New Zealand
Uchida *et al.* (2011)	590 (lab trial)	*Lolium perenne* L.	-	1.45-6.43	0.18-0.88	-	0.2-2.2	-	-	28	Clay loam	New Zealand
Taghizadeh-Toosi (2011)	930 late spring	*Lolium perenne* L.	-	17.6	-	-	0.86	-	-	86	Silt loam	New Zealand
Shepherd *et al.* (2010)	500 late winter	*Lolium perenne* L. + *Trifolium repens* L.	15	48.1	-	18.9	-	-	-	247	Silt loam	New Zealand
Wachendorf *et al.* (2008)	1030 autumn	*Lolium perenne* L. dominated	53.4	0.3	-	12.8	0.05	-	-	171	Sand over gravel	Germany
Ambus *et al.* (2007)	510 spring	*Lolium perenne* L. + *Trifolium repens* L.	-	~15	-	~30	≤ 0.3	-	-	41	Sandy loam	Denmark
Silva *et al.* (2005)	1000 autumn	*Lolium perenne* L. + *Trifolium repens* L.	6	39	19	28	-	-	2	365	Fine sandy loam	New Zealand
Decau *et al.* (2004)	165 autumn 165 spring	*Lolium perenne* L.	18.3-25.9 0.1-14.1	-	-	-	-	-	-	730	Clay loam over loam	France

	Urine rate		% of applied N recovered									
Study	**(kg N ha^{-1})**	**Plant type(s)**	**Leaching**	**Forage**		**Soil**	**Denitrification**		**NH_3**	**Days**	**Soil texture**	**Country**
Bol *et al.* (2004)	398 (Urea) autumn	*Lolium perenne* L. + *Trifolium repens* L.	-	>1	-	47	0.015	-	12	14	Sandy loam	Denmark
Leterme *et al.* (2003)	spring	*Lolium perenne* L.	-	57-62.4	2.4-2.6	23-26.6	-	-	1.3	350	Silt loam	France
	summer			46.1-55.3	4-5.9	26.8-30.5			2.6			
	autumn			21.4-35.9	8.9-12.8	26.5-28.1			1.62			
Decau *et al.* (2003)	520											
	spring		0.7	58		26						
	summer		7.7	42.3		29.7						
	autumn		16.7	32		30.3						
		Lolium perenne L.	9	36.7	-	30.7	-	-	-	730	Clay loam over loam	France
			14.7	46.3		30.7					Loam	
			1.3	49.3		24.7					Silt loam	
Clough *et al.* (2003)	500 (lab trial)	-	-	-	-	-	0.82	-	-	60	Silt loam	New Zealand
Di *et al.* (2002)	1000 autumn	*Lolium perenne* L. + *Trifolium repens* L.	6.4	38.8	19.2	28.3	-	-	2[2]	365	Fine sandy loam	New Zealand
Clough *et al.* (2001)	560 (as KNO_3) lab trial	None	26.69	-	-	43.9	2.18 9.32[3]	1.45 13.28[3]	-	20	Silt loam	New Zealand
Williams and Haynes (2000)	193 spring	*Lolium perenne* L. + *Trifolium repens* L.	-	~50	-	~20-30	-	-	-	365	Silt loam	New Zealand
Thompson and Fillery (1998)	123-259 spring summer autumn	*Triticum aestivum* L.	0-20	4-37	-	22-37	0-10		10-50	370-410	Loamy sand	Australia
Clough *et al.* (1998)			31.7	22.7	0.07	20.6	0.8		0.7		Silt loam	
	1000 winter	*Lolium perenne* L. + *Trifolium repens* L.	13.1	31.4	0.07	23.0	1.0	-	2.3	406	Sandy loam	New Zealand
			18.0	21.6	0.05	24.3	1.9		2.4		Peat	
			23.5	28.8	0.04	23.3	1.9		3.9		Clay	

	Urine rate		% of applied N recovered									
Study	**(kg N ha^{-1})**	**Plant type(s)**	**Leaching**	**Forage**		**Soil**	**Denitrification**		**NH_3**	**Days**	**Soil texture**	**Country**
Clough *et al.* (1996)	500 early winter	*Lolium perenne* L. + *Trifolium repens* L.	6.1-51.8	8.1-25.8	2.8-6.8	20.9-23.8	-	-	-	150	Silt loam	New Zealand
Sorensen and Jensen (1996)	204	*Lolium multiflorum* L.	-	61.2-69.3	-	12.7-15.5	-	-	-	150	Sandy loam	Denmark
Pakrou and Dillon (1995)	1093 winter	*Lolium perenne* L. + *Trifolium repens* L. (irrigated).	62	3		14				84	Sandy loam	Australia
	888 spring		13-41	13-31		20-31				297		
	1366 summer		54	8		26				80		
					-		-	-	-			
	1093 winter	*Trifolium subterraneum* L. + annual grasses (non-irrigated).	51	20		23				84		
	888 spring		10-41	16-23		23-44				297		
	1366 summer		25	-		63				80		
Fraser *et al.* (1994)	500 winter	*Lolium perenne* L. + *Trifolium repens* L.	8.3	43.5		20.4	-	-	-	365	Silt loam on sandy loam	New Zealand
McLaren *et al.* (1993)	500 winter	*Lolium perenne* L. + *Trifolium repens* L.	15.9	39.6		25.9	-	-	-	365	Silt loam	New Zealand
Whitehead and Bristow (1990)	744 summer	*Lolium perenne* L.	-	20.1	2.7	17.4	6.7		17.8	321	Clay loam	England
Vallis *et al.* (1985)	150 late winter	*Alysicarpus vaginalis, Brachiaria* spp., *Digitaria* spp. + *Urochloa mosambicensis* then *Sorghum bicolor* L. crop	-	8-8.6		19.7-21.2	-	-	-	237	Clay loam	Australia
Keeney and Macgregor (1978)	300 late summer	*Lolium perenne* L. + *Trifolium repens* L.	-	19.4	5.6	63.7	-	-	-	22	Silt loam	New Zealand

[1]No data; [2]Estimate; [3]Glovebox gas from soil core destructive sampling

2.4 Synthesis of literature review findings and hypotheses

The review of the literature has identified the following key knowledge gaps:

- Emerging research indicates that Italian ryegrass may reduce NO_3^- leaching from grazed agricultural soil, however more information is required to understand the mechanisms involved and to quantify the effects more accurately.
- Although lucerne has been described as having the potential to reduce N leaching from grazed systems (through its ability to extract water and N from depth via its deep root system) there are no studies which measured NO_3^- leaching losses from lucerne under a grazed dairy system/animal urine patch.
- Forages containing plantain can reduce urine-N excretion, which many researchers have suggested could reduce N leaching losses. However, no studies have measured N leaching losses from forages containing plantain which take into account actual urine-N excretion rate by the grazing animals.
- The literature review has indicated that an application of gibberellic acid could potentially increase the uptake of urine-N deposited in autumn, due to its ability to increase forage DM production in the shoulders of the season, when forages would otherwise be limited by cool temperatures. This could subsequently reduce N leaching, due to less N remaining in the soil prior to the drainage occurring in winter. However, no studies were found which investigated the effect of GA application to forages on NO_3^- leaching.
- Nitrogen-^{15}N balance studies provide robust data and can trace the fate of urine-N within a particular system. Most reported ^{15}N balance studies have been conducted on perennial ryegrass-white clover. There is a need for more ^{15}N balance studies for alternative forages in order to better understand the N cycling and losses in these systems.

The following research chapters address these knowledge gaps through testing of the following hypotheses:

1. That alternative forages such as Italian ryegrass and lucerne reduce N leaching compared with that of typical perennial ryegrass-white clover forage through mechanisms such as increased winter activity and root depth.
2. That gibberellic acid applied to forage in autumn increases both herbage growth and the uptake of urinary-N, subsequently reducing N leaching losses.
3. That an increase in the uptake of urinary-N by plants reduces the amount of urinary-N leached.
4. That Italian ryegrass decreases N leaching by inhibiting the first step of the nitrification process: ammonia oxidation.

5. That an Italian ryegrass-plantain-white clover mixture would have a lower leaching loss than perennial ryegrass-white clover.
6. That cows grazing the Italian ryegrass-plantain-white clover mixture have lower urine-N excretion, compared with perennial ryegrass-white clover.
7. That the Italian ryegrass-plantain-white clover mixture would take up more N during the cool season than perennial ryegrass-white clover.
8. That the application of GA to perennial ryegrass-white clover reduces N leaching from urine patches in autumn, but that there is a maximum urine-N rate above which this effect is negligible.

Chapter 3

Lysimeter Experiment 1

3.1 Introduction

Nitrate leaching is a significant environmental concern in intensively grazed New Zealand forage-based systems. Elevated levels of NO_3^- in surface waters can cause eutrophication, where algal blooms and excessive plant growth consume oxygen, causing other aquatic life to die (Howarth, 1988; Smith & Schindler, 2009). This represents a significant decline in water quality. If NO_3^- is leached into drinking water supplies, this is considered a risk to human health when concentrations exceed 11.3 mg NO_3^--N L^{-1} (equal to 50 mg NO_3^- L^{-1}) (WHO, 2011).

Animal urine patches are the main source of NO_3^- leaching loss in forage-based systems where grazing occurs outdoors year-round. This is because the animals only use a small proportion (5-30%) of the nitrogen they ingest and the remaining 70-95% is excreted in dung and urine (Oenema *et al.*, 2005). The majority (~60%) of this excreted N is deposited as highly concentrated urine patches (Haynes & Williams, 1993) (average 613 kg N ha^{-1}, range 200-2000 kg N ha^{-1} from Selbie *et al.* (2015)) which may cover 20-30% of a grazed field annually depending on the stocking density (Moir *et al.*, 2011). These urine-N loading rates result in an input of nitrogen into the soil-plant system which often exceeds plant requirements. The N which is not taken up by the forage is often leached from the soil as NO_3^- in drainage water (Cameron *et al.*, 2013) where it can contaminate ground and surface waters. Thus the development of mitigation methods to reduce N leaching losses from these farm systems are urgently required.

Nitrate leaching processes, factors affecting leaching, and methods to reduce NO_3^- leaching have been thoroughly reviewed by Cameron *et al.* (2013). One approach to mitigation is to increase the uptake of N by the forage, particularly during the cooler seasons when the risk of leaching is greatest. If plants can utilise urine-N more efficiently at these times of year, N losses to drainage water could be reduced. The rate of plant growth and size of the root system have been shown to be important regulators of NO_3^- interception in ryegrass (*Lolium* sp.), with root depth of secondary importance (Crush *et al.*, 2007). Additionally, NO_3^- leaching has been linked to winter daily N uptake by pasture (Malcolm *et al.*, 2014). Currently, perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) mixtures are common forages in grazed dairy systems in New Zealand. Similarly, lucerne (*Medicago sativa* L.) a high yielding, deep rooting, drought tolerant summer forage is also autumn-grazed in some systems. However, these species do not grow well over the winter period, when temperatures are cooler, and

supplements or winter forages are often utilised to meet animal feed demands. An improved understanding of the susceptibility of these forages, and winter-active alternatives, to potential N loss via leaching and related plant N uptake will allow for better management and increase the efficiency of N use in agricultural systems. This is critical in minimising N losses. Using ^{15}N to trace the fate of urinary-N provides robust data for improving understanding of the N cycle within a particular system and associated N losses. A number of ^{15}N balance studies have traced the fate of urine within forage systems (Table 2.1), however, these have been predominantly performed on either perennial ryegrass monocultures or perennial ryegrass and white clover mixtures.

To enhance plant uptake of N and thus potentially reduce the risk of N leaching, the use of gibberellic acid, a plant growth stimulant, has been proposed (Parsons *et al.*, 2013). Gibberellic acid is responsible for stem elongation and leaf expansion in plants (Matthew *et al.*, 2009). It has been shown to increase pasture dry matter production in the shoulders of the season when cool temperatures limit plant growth (Matthew *et al.*, 2009). This extra growth, when the forage plants would otherwise be growing very slowly, could potentially increase the uptake of urine deposited N. Although previous studies have shown decreased herbage-N or crude protein (CP) content following an application of GA (Scurfield, 1958; Finn & Nielsen, 1959; Biddiscombe *et al.*, 1962; McGrath & Murphy, 1976; Percival, 1980; Ghani *et al.*, 2014), increased herbage-N or CP yield have also been reported (Morgan & Mees, 1958; Finn & Nielsen, 1959; Biddiscombe *et al.*, 1962). No studies have measured the effect of GA on N leaching.

Therefore, the objectives of this experiment were to quantify the effects of forage type and GA application on N leaching and herbage N uptake, under autumn-deposited ruminant urine patches, and to determine the fate of the urine-N applied for each of the treatments using a ^{15}N tracer.

This experiment tested the following key hypotheses:

1. That alternative forages such as Italian ryegrass (*Lolium multiflorum* Lam.) and lucerne reduce N leaching compared with that of typical perennial ryegrass-white clover forage through mechanisms such as increased winter activity and root depth.
2. That gibberellic acid applied to forage in autumn increases both herbage growth and the uptake of urinary-N, subsequently reducing N leaching losses.
3. That an increase in the uptake of urinary-N by plants reduces the amount of urinary-N leached.

3.2 Methodology

3.2.1 Experiment description and preparation

The soil type (Plate 3.1) was described as a Paparua sandy loam by E. J. Cutler in 1971 (Figure A 1) and by Kear *et al.* (1967). These soils are now correlated at the national level to the Barrhill family, sibling

number 5 (Landcare Research, 2015) and are described as deep, loam over sand, stoneless soils which are well drained and have high profile available water. However, site-specific texture analysis and profile description indicated that the soil was more closely correlated to the Templeton family, as the sand layer was not the dominant texture over the 0 to 60 cm depth, which is the criteria for the Barrhill family (S. Carrick, personal communication, July 23, 2015). In the New Zealand Soil Classification these are classified as Typic Immature Pallic soils (Hewitt, 2010); USDA: Udic Haplustept, (Soil Survey Staff, 2014). Soil fertility tests were conducted to determine nutrient status and pH of the soil prior to the start of the experiment (Table 3.1). This helped determine nutrient requirements of the different forages. Soil was sampled to 7.5 cm depth using a corer at multiple sites in the fields from which the lysimeters were collected. This soil was then sent to a commercial laboratory for analysis (Analytical Research Laboratories, NZ).

Soil was air dried at 38°C and ground to 2 mm prior to all analyses. Soil pH was measured following equilibration for 1-4 hours at a soil:water of 1:2.5 (Blakemore *et al.*, 1987). Olsen P was determined by extracting the soil with 0.5 M $NaHCO_3$ (pH 8.5), for 30 minutes at a soil:water ratio of 1:20. Orthophosphate-P in the extracts were then determined colorimetrically using a Lachat Flow Injection Analyser (Olsen *et al.*, 1954; Murphy & Riley, 1962; Blakemore *et al.*, 1987). Organic matter, total C, and total N levels were determined using an Elementar Vario Max Cube Analyser. Sulphate-S was determined by extraction with 0.02 M KH_2PO_4 for 30 minutes at a soil:solution ratio of 1:10, followed by ion chromatography with sodium hydroxide as eluent (Watkinson & Kear, 1994). Soil cations (K^+, Ca^{2+}, Mg^{2+}, and Na^+) were determined by first shaking the soil with 1 M ammonium acetate (pH 7) for 30 minutes, at a soil:solution ratio of 1:20. These were filtered and analysed by either Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) or Microwave Plasma Atomic Emission Spectroscopy (MP-AES), this method was modified from Rayment and Higginson (1992). Cation exchange capacity (CEC) was determined by summing these exchangeable cations plus hydrogen, which was determined by change in pH (Brown, 1943).

Table 3.1 Soil fertility test (0-7.5 cm) and soil particle size (depths: 0-15, 15-30, 30-45, 45-65 cm) results from the fields where lysimeters were collected.

	Perennial ryegrass-white clover and Italian ryegrass	Lucerne
pH	5.7	6.3
Olsen P (μg g^{-1})	23.1	42.5
Organic Matter (g kg^{-1})	40	
Total C (g kg^{-1})	23.3	
Total N (g kg^{-1})	1.9	
Sulphate S (μg g^{-1})	34	49
CEC[1] ($cmol_c$ kg^{-1})	12	12
Exchangeable Ca^{2+} ($cmol_c$ kg^{-1})	6.1	7.6
Exchangeable Mg^{2+} ($cmol_c$ kg^{-1})	0.34	1.05
Exchangeable K^+ ($cmol_c$ kg^{-1})	0.31	1.16
Exchangeable Na^+ ($cmol_c$ kg^{-1})	0.18	0.16
BS[2] (%)	55.8	83.3
Sand-silt-clay (%)		
0-15 cm	64-27-8	72-21-7
15-30 cm	67-25-8	75-18-7
30-45 cm	77-19-4	78-18-4
45-65 cm	84-14-2	84-13-2

[1]Cation exchange capacity; [2]Base saturation

Field history

The perennial ryegrass-white clover field was sown in April 2011 and had previously been rotationally grazed by Friesian-Jersey-cross cows as part of a farmlet experiment. The lucerne field had previously been grazed perennial ryegrass-white clover. The field was sprayed with glyphosate, ploughed, cultivated, and lucerne was sown on 15 February 2012 at a rate of 10 kg seed ha^{-1} using a roller drill. The lucerne was managed by grazing with Friesian-Jersey-cross cows though it was often cut ahead of the cows. Weeds were controlled chemically. Haloxyfop-P (250 mg 100 L^{-1} water) was applied in April 2012 to control *Poa annua* sp. and Flumetsulam (65 g ha^{-1}) was applied one month later to control broadleaf weeds. In August 2013 Simazine (1.5 L 100 L^{-1} water) was applied to control spring broadleaf weed germination.

Plate 3.1 Paparua sandy loam soil at the lysimeter collection site on the Lincoln University Research Dairy Farm.

3.2.2 Lysimeter collection and installation

In November-December 2013, 45 lysimeters (0.5 m diameter, 0.7 m deep) were collected from Lincoln University Research Dairy Farm using well-established protocols and procedures described in Cameron *et al.* (1992). In summary, this involved placing a metal casing on the soil surface, digging the soil around it, and gradually pushing the casing down in small increments to 0.68 m. A cutting plate was used to cut the soil monolith. This was then secured onto the lysimeter casing and lifted out of the collection site. A free-draining condition similar to that in the field was created by replacing the bottom 0.05 m of soil with gravel. Care was taken not to disturb the soil structure inside. Petroleum jelly was used to seal the gap between the soil core and the metal casing to stop edge-flow effects (Plate 3.2).

Plate 3.2 Lysimeter collection where: a) shows how the soil is dug around the outside of the lysimeter casings, b) the casing is tapped down in small increments, c) the edges are sealed with Petroleum jelly, d) a hydraulic ram is used to insert the cutting plate beneath the lysimeter, and e) lysimeters have been lifted from the pit, gravel replaces the bottom 0.05 m of soil and base plates are fitted.

The lysimeters were transported to Lincoln University's Field Research Centre on a specially designed trailer with air-bag suspension to minimise disturbance. They were then installed in a trench facility at the same level as the surrounding field using a tractor to carefully lower them into position (Plate 3.3). Plastic tubing was connected to the base of each lysimeter and fed into a 10 L container for leachate collection. Lysimeters were then levelled and soil was backfilled to the same level as the surrounding field (Plate 3.4).

Plate 3.3 Installation of lysimeters in the trench facility at Lincoln University's Field Research Centre: a) transportation of lysimeters from the trailer to the trench, and b) carefully lowering the lysimeters into position in the trench.

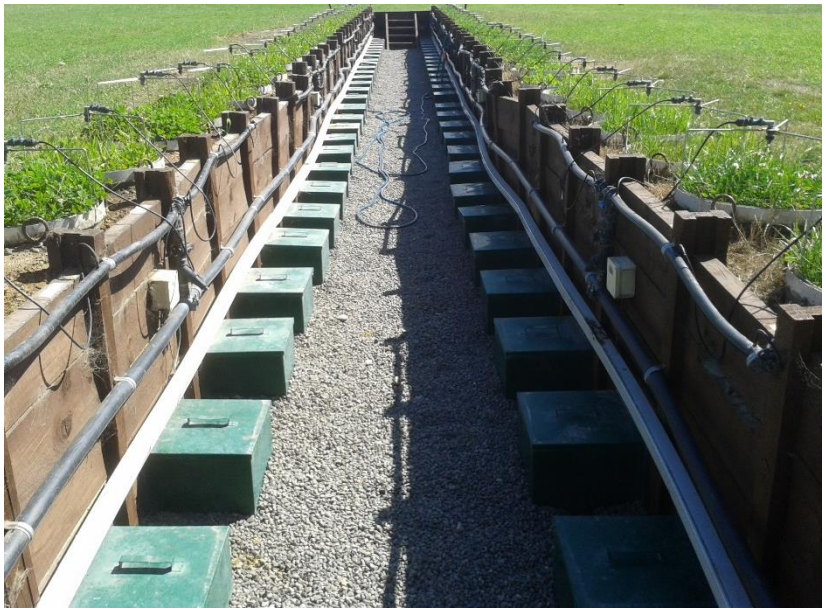


Plate 3.4 Lysimeters installed in the trench facility at Lincoln University's Field Research Centre.

3.2.3 Treatments and experimental design

Treatments

Lysimeter treatments are summarised in Table 3.2. The experiment consisted of nine treatment combinations, replicated five times. Fifteen of the lysimeters were collected from a field of lucerne (*Medicago sativa* L.) cv. 'Force 4' (Seed Force), and 30 from a perennial ryegrass (*Lolium perenne* L.) and white clover field (*Trifolium repens* L.) (RGWC). The perennial ryegrass cultivar in this field was Expo with AR1 endophyte, and the white clover cultivar was 'Kopu II' (PGG Wrightson Seeds). On 20 February 2014, 15 of the RGWC lysimeters were sprayed with glyphosate (20 mL glyphosate in 2 L) using a knapsack sprayer. Treatment shields were used to avoid spray drift. On 7-10 March 2014 these lysimeters were cultivated (to 0.05 m) using hand gardening tools to provide a fine seed bed. Italian ryegrass (*Lolium multiflorum* Lam.) cv. 'Tabu' (Agriseeds) was sown into these lysimeters at a rate of 25 kg ha^{-1} (equal to 0.5 g per lysimeter) on 12 March 2014 (Plate 3.5) (Italian RG).

Table 3.2 Lysimeter treatments.

Treatment no.	Forage type	Treatment	Replication	Cultivar
T1	Perennial ryegrass + white clover (RGWC)	Control	5	Expo (AR1) + Kopu II
T2	Perennial ryegrass + white clover (RGWC)	Urine	5	Expo (AR1) + Kopu II
T3	Perennial ryegrass + white clover (RGWC)	GA[1] + Urine	5	Expo (AR1) + Kopu II
T4	Italian ryegrass (Italian RG)	Control	5	Tabu
T5	Italian ryegrass (Italian RG)	Urine	5	Tabu
T6	Italian ryegrass (Italian RG)	GA + Urine	5	Tabu
T7	Lucerne	Control	5	Force 4
T8	Lucerne	Urine	5	Force 4
T9	Lucerne	GA + Urine	5	Force 4

[1]Gibberellic acid

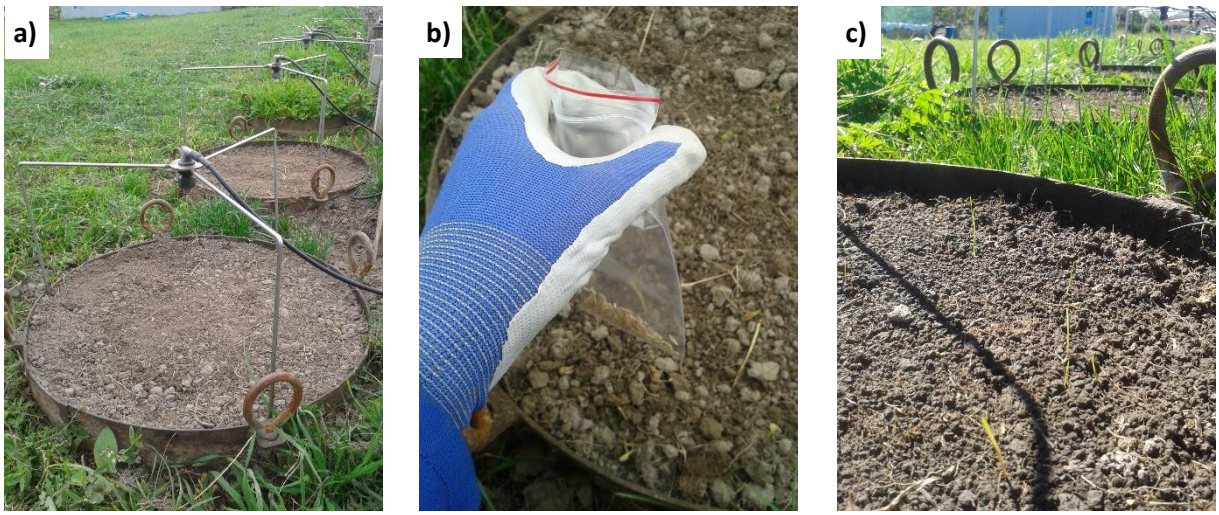


Plate 3.5 Sowing of Italian ryegrass to lysimeters: a) the prepared seed bed, b) seeds being sown, and c) seedlings emerging from soil.

Experimental design

The experiment was a split-plot design with forage type as main plots, and treatment as sub-plots, laid out in five replicate blocks as illustrated in Figure 3.1. Forage type was randomised within replicate blocks, and treatments randomly allocated to lysimeters within the main plots using Genstat (16th Edition, VSN International Ltd.).

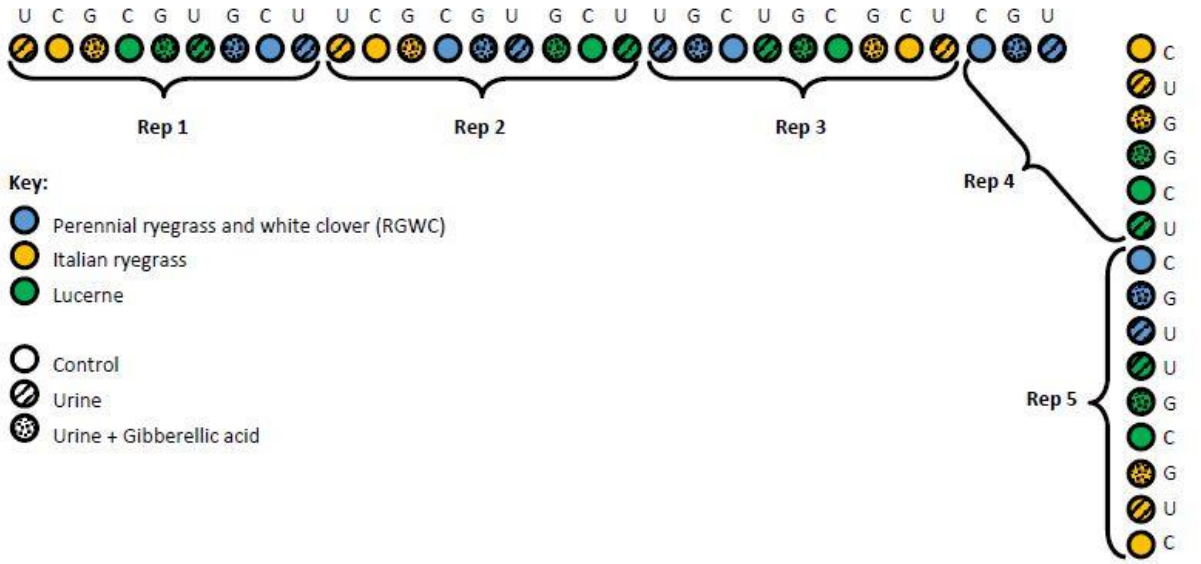


Figure 3.1 Lysimeter experiment layout.

Treatment application

On 5 May 2014, herbage on all lysimeters was harvested to a residual height of 0.05 m using an electric shearing hand piece. Fresh cow urine (>70 L) was collected during the afternoon milking from Friesian-Jersey-cross (KiwiCross™) cows which had been grazing perennial ryegrass-white clover at the Lincoln

University Dairy Farm. A urine sample was collected, and was analysed overnight for N concentration on an Elementar Vario-Max CN Elemental Analyser (Elementar GmbH, Hanau, Germany). The urine was found to have a N concentration of 5.02 g N L^{-1}. The day after collection, the urine was labelled with ^{15}N by adding highly enriched ^{15}N urea (98 atom%; Cambridge Isotope Laboratories, Inc.), and unlabelled urea and glycine (9:1 ratio) so that the N concentration increased to 7 g N L^{-1} with a ^{15}N enrichment of 5 atom%. The glycine was used to represent the amino acid fraction of urine and better mimic the actions of real urine (Fraser *et al.*, 1994). The urine was mixed thoroughly until the urea and glycine had dissolved, 2 L was then measured out using a volumetric flask and applied to each appropriate lysimeter to simulate urine patches deposited by grazing dairy cows (Plate 3.6). This represented a rate of 700 kg N ha^{-1} which is typical of a cow urine patch (average 613 kg N ha^{-1}, range 200-2000 kg N ha^{-1} from Selbie *et al.* (2015)). Control plots received 2 L of water so that moisture inputs would remain consistent.

Plate 3.6 Application of urine treatments to lysimeters.

The next day gibberellic acid solutions (8 g GA ha^{-1}, 50 mL surfactant ha^{-1}) were prepared by serial dilution. Firstly 1 g of ProGibb®SG (containing 40% gibberellic acid, Valent BioSciences Corporation, IL, USA, marketed by Nufarm Ltd., New Zealand) was weighed out, added to a 0.2 L volumetric flask, and stirred until dissolved. Next, 2.5 mL of surfactant (Spreadwet 1000, active constituent: 1000 g L^{-1}

alkoxylated alcohols, SST NZ Ltd.) was added and the flask made up to 0.2 L volume with deionised water. A 10 mL aliquot was taken from this flask after it was mixed well. This was put into a 0.5 L volumetric flask and made up to volume. A second solution was made up in the same manner, but contained only the surfactant. In the field, 4 mL of the gibberellic acid solution was pipetted into the chamber of a small, battery powered, airbrush sprayer (Spray-Work Basic Air Compressor w/Airbrush, Tamiya, Inc., Shizuoka City, Japan) (Plate 3.7). This was applied evenly to the appropriate lysimeters as a fine mist. Treatment shields (~30 cm high) were used to prevent spray drift. All other lysimeters (non-GA treated) received an application of 4 mL of the surfactant-only solution using the same application technique. The 8 g GA ha^{-1} rate of GA used is the commercial rate, and previous studies have shown this to give a dry matter (DM) response (Matthew et al., 2009; Jiang et al., 2011; Ball et al., 2012).

Plate 3.7 Application of gibberellic acid to lysimeters using an airbrush sprayer.

3.2.4 Lysimeter maintenance

Fertiliser applications

Lysimeters received maintenance fertiliser on 15 April 2014 prior to treatment application. The RGWC and Italian RG lysimeters all received 200 kg ha^{-1} of superphosphate (0:9:0:11) and the lucerne lysimeters received 300 kg ha^{-1} of Lucerne Mix (0:6:15:13) (Table 3.3).

Nitrogen was applied as urea (on 28 April 2014) to all lysimeters at a rate of 25 kg N ha^{-1} prior to treatment application to encourage initial growth of the young Italian ryegrass seedlings and to avoid N deficiencies in Control plots. To simulate standard dairy farm practice, further applications of urea, at the same N rate, post-cutting were made to RGWC and Italian RG on 9 October 2014, 7 November

2014, 18 December 2014, 15 January 2015, 13 February 2015, and a final application of 20 kg N ha^{-1} on 12 March 2015 (Table 3.3).

Again to simulate typical dairy farm practice, ammonium sulphate (NH_4SO_4; 21:0:0:24) was applied to the RGWC and Italian RG lysimeters on 21 August 2014 at a rate of 30 kg N ha^{-1}. Gypsum ($CaSO_4$; 0:0:0:18) was applied to the lucerne lysimeters on the same date at the same rate of sulphur (34 kg S ha^{-1}). This provided sulphate to the lucerne but not ammonium due to its ability to fix its own N, lucerne did not require the N from the ammonium.

Table 3.3 Fertiliser applications over the 17-month experimental period.

Product	Rate	RGWC	Italian RG	Lucerne
Superphosphate	18 kg P ha^{-1}	15/4/2014	15/4/2014	-
	22 kg S ha^{-1}	4/9/2015	4/9/2015	
Lucerne mix	18 kg P ha^{-1}			15/4/2014
	45 kg K ha^{-1}	-	-	4/9/2015
	39 kg S ha^{-1}			
Urea	25 kg N ha^{-1}	28/4/2014	28/4/2014	
		9/10/2014	9/10/2014	
		7/11/2014	7/11/2014	
		18/12/2014	18/12/2014	28/4/2014
		15/01/2015	15/01/2015	
		13/2/2015	13/2/2015	
		4/9/2015	4/9/2015	
Urea	20 kg N ha^{-1}	12/3/2015	12/3/2015	-
Ammonium sulphate	30 kg N ha^{-1}	21/8/2014	21/8/2014	-
	34 kg S ha^{-1}			
Gypsum	34 kg S ha^{-1}	-	-	21/8/2014
	44 kg Ca ha^{-1}			
Magnesium oxide	25 kg Mg ha^{-1}	30/1/2015	30/1/2015	30/1/2015
Total fertiliser-N	(kg N ha^{-1})	225	225	25

Magnesium oxide (MgO; 52% Mg) was applied to all lysimeters on 30 January 2015 at a rate of 25 kg Mg ha^{-1} to alleviate observed magnesium deficiencies following herbage testing through a commercial laboratory (Analytical Research Laboratories, NZ) (Table C 1,2).

Pest and weed control

Slug bait (McGregor's snail and slug pellets) was sprinkled on the field area immediately outside of the lysimeters on 29 May 2014, to help control slugs in the lysimeters during the winter period. Yates Soil Insect Killer (50 g kg^{-1} diazinon) was applied to lysimeters on 26 February 2015 to again control slugs.

Weeds were controlled within the lysimeters initially by painting glyphosate onto the target weed species, later in the experiment they were hand weeded but residuals of the weeds were left within the lysimeter area. Lucerne lysimeters received an application of Gallant Ultra (1 mL L^{-1} with 5 mL L^{-1} of wetting agent: straight uptake oil) on the 27th June 2014 to help control grass weeds. This was applied using a solo knapsack (15 L) with a Tee-Jet. Treatment shields were used to prevent drift.

Other

Frost cloth was placed over the entire experiment periodically from 26 May 2014 to 3 June 2014 when frost was predicted. This was to prevent potential frost damage to lucerne plants, and ensure enough plant material for the first post-treatment herbage harvest.

3.2.5 Rainfall and irrigation simulation

Lysimeters were fitted with an automated sprinkler system. Each lysimeter had a spray nozzle (Tee Jet FL-5VC) mounted directly over the top of it (Plate 3.8). Water was applied to lysimeters either as simulated rain or irrigation (Appendix B, Figure B 2-5). A detailed description of the system can be found in Appendix B. In brief, rainfall was supplemented with randomly simulated rain based on a daily target. Targets were primarily based on historical and daily climate data and driven by a CR 3000 Campbell Scientific data logger. To account for evapotranspiration and prevent soil moisture deficits during the drier period of the year (October to March), irrigation was applied to lysimeters at regular rates and intervals.

Additional climate information was also recorded at the lysimeter trench facility. Rainfall was measured using a TB3 Tipping Bucket Rain Gauge. Ground (at 10 cm depth) and air temperature were determined by 107 Campbell Scientific temperature sensors. Wind speed was recorded using a Maximum Type 40 Anemometer. A platinum resistance thermometer (PT100) was used to monitor water temperature in the pipes of the irrigation system and to monitor temperature at the soil surface to indicate the occurrence of frosts.

Plate 3.8 Lysimeter sprinkler system showing spray nozzle mounted above lysimeter.

3.2.6 Nitrate leaching measurements

Leachate collection

Preliminary leachate samples were collected on three occasions to determine the initial NH_4^+ and NO_3^- concentrations in the leachate. Throughout the experiment, leachate was collected from each lysimeter when rainfall or irrigation caused drainage above 200 mL, or at least once a week. Drainage volume was recorded using plastic measuring jugs, and two "mid-stream" leachate samples were collected in 50 mL plastic bottles (Plate 3.9). Samples were kept frozen (-20°C) until analysis.

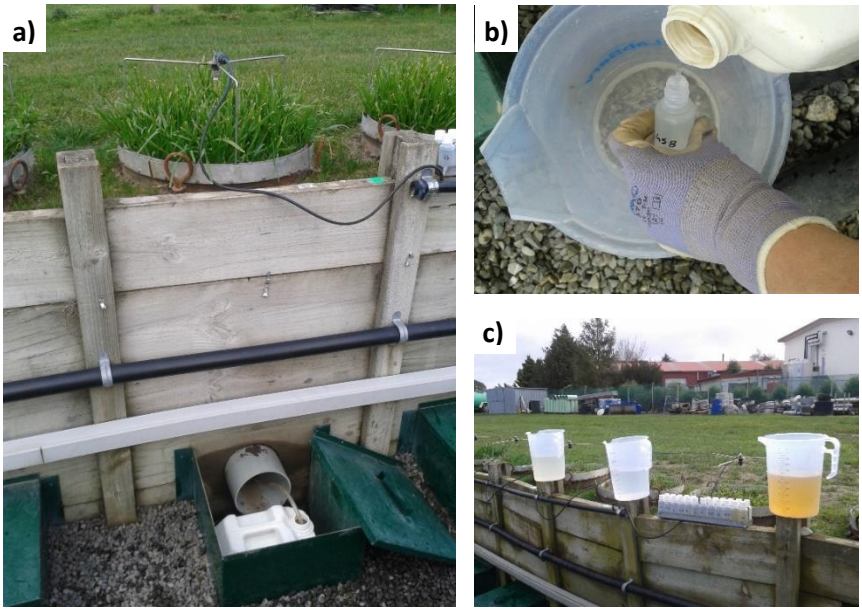


Plate 3.9 Leachate collection from lysimeters: a) lysimeter collection container setup, b) leachate sample being collected, and c) drainage volume recorded in plastic measuring jugs.

Analysis of leachate

Leachate samples were analysed for NO_3^--N and NH_4^+-N concentrations by flow injection analysis (FIA) using a FOSS FIAstar 5000 twin channel analyser (Foss Tecator AB, Hoganas, Sweden). Nitrate-N was analysed by initial reduction of NO_3^--N to NO_2^--N using a cadmium reduction coil (OTCR - open tubular cadmium reactor). This was then reduced with sulphanilamide/NED to form an azo dye compound. The intensity of this compound was determined spectrophotometrically at 540 nm.

Ammonium-N was determined using a gas diffusion membrane. Sodium hydroxide was added to increase the pH of the sample stream, and any NH_4^+ ions present were converted into ammonia gas. This then diffused through the membrane into an indicator stream which changed colour (red to blue) with an increase at 590 nm. The extent of the colour change was proportional to the concentration of NH_4^+ ions present in the sample.

At a later date, ^{15}N enrichment of total N in selected leachate samples was determined by continuous flow isotope ratio mass spectroscopy (IRMS) (Sercon Ltd, Crewe, CW1 6JT, UK). Samples were prepared using the diffusion procedure described by Brooks *et al.* (1989). In brief this involved the reduction of NO_3^- to NH_4^+ and then conversion of this and any NH_4^+ from the sample to ammonia. This ammonia diffused into the headspace of the sealed sample container where it was collected on acidified 7 mm glass fibre filter paper disks (MicroScience MS GD, 47 mm) suspended on a wire above the liquid sample in the sealed container, over 6 days (Figure 3.2). The filter papers were first cleaned by washing in 2M KCl solution three times, followed by deionised water three times then dried and punched into 7 mm disks. These disks were acidified with 10 µL of 2.5 M $KHSO_4$ no more than 5 minutes before capping the container.

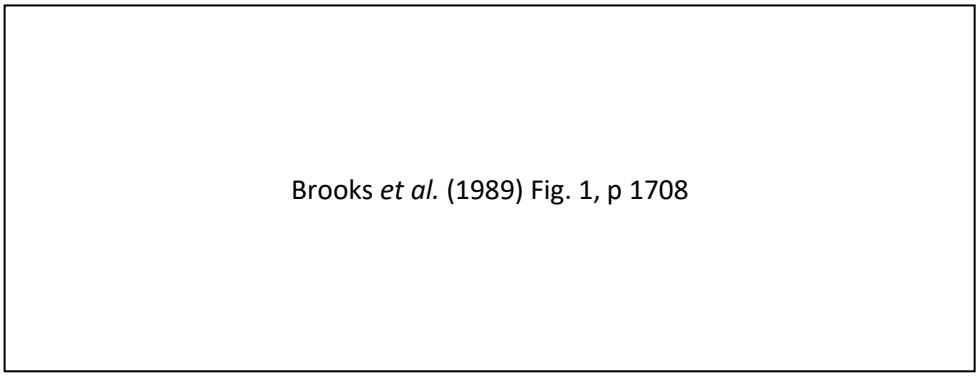


Figure 3.2 Diffusion apparatus to prepare leachate samples for ^{15}N analyses. From Brooks *et al.* (1989).

The volume of the leachate sample diffused varied, depending on the NO_3^--N and NH_4^+-N concentrations (previously determined by FIA), so that there was 50-120 µg of N on the disk. Deionised water was added to make the total volume 50 mL. Next, 0.1 mL of 21% w/v Brij-35 solution, 0.4 g of

Devarda's alloy, and 0.2 g of MgO were added. The container was immediately capped and gently mixed with the help of two acid-washed glass beads in the container, care was taken not to splash the solution onto the disk. After 6 days the disks were dried in a desiccator for at least 24 hours, and then sealed in tin capsules ready for IRMS analysis. Deionised water blanks and two standards were included in each run and were replicated three times.

3.2.7 Forage production measurements

Perennial ryegrass-white clover and Italian ryegrass

The RGWC and Italian RG lysimeters were harvested once plant development had reached the 2-3 leaf stage (Figure 3.3) and yields were on average 3000 kg DM ha^{-1}. An electric shearing hand piece (Plate 3.10) was used to cut herbage to a residual height of 50 mm (approx. 1500 kg DM ha^{-1}). These pre and post grazing DM residuals are typical of management practices on New Zealand dairy farms throughout the season.

All of the plant material was collected in paper bags. Fresh weight (FW) was recorded, then herbage samples were oven-dried at 65°C for at least 48 hours, to determine dry weight (DW). Dry matter content (%) was then calculated as DM% = DW/FW x 100. Herbage samples were then stored until they were ground. The night before grinding, they were put back into the oven to remove any moisture which may have been absorbed into the samples during storage. Samples were then ground using a Retsch Ultra Centrifugal Mill ZM 200, with a 1 mm sieve and running at a speed of 18,000 rpm. Care was taken to thoroughly clean the grinder in-between each sample. Ground plant material was stored in sealed 70 mL containers at room temperature in the dark, ready for further analysis.

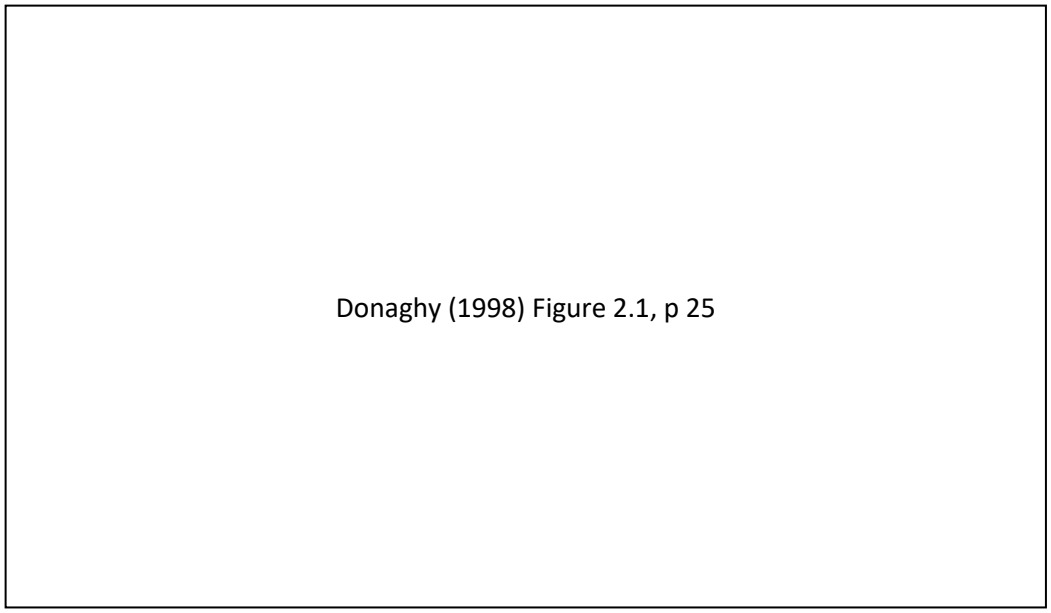


Figure 3.3 Diagram showing the three-leaf stage of a grass plant (modified from Donaghy, 1998).

Plate 3.10 Herbage is harvested using an electric hand piece, or hand clippers.

Lucerne

Lucerne lysimeters were harvested using an electric hand piece or hand clippers to a residual height of 50 mm according to the following seasonal management information (Moot *et al.*, 2003):

Autumn (Feb-Mar): allow at least 50% of the lucerne stems to have an open flower sometime between mid-summer to autumn to encourage root recharge. Long rotation.

Winter (June-July): Final hard graze early winter (first week of June). Leave to develop new shoots until spring.

Spring (Aug-Nov): Graze when herbage 20-25 cm high (1500 kg DM ha^{-1}), allow 5-6 weeks regrowth (35-45 cm height, ~3000 kg DM ha^{-1}).

Summer (Dec-Jan): 30-35 day rotation.

Samples were harvested, dried and ground using the same technique as for the RGWC and Italian RG lysimeters.

Analysis of herbage

Ground plant samples were analysed for total N content using an Elementar Vario-Max CN Elemental Analyser (Elementar GmbH, Hanau, Germany). At a later date, ground herbage samples were weighed into tin capsules, and the ^{15}N enrichment was determined by IRMS for six of the 14 batches collected. Forage quality parameters such as organic matter (OM), water-soluble carbohydrate (WSC), crude protein (CP), and dry matter digestibility (DMD) concentration were determined using near infra-red spectroscopy (NIRS) (Model: FOSS NIRSystems 5000; FOSS NIRSystems Inc., MD USA). Metabolisable energy (ME) was calculated using the equation:

$$ME = (DMD + 3) \times OM/100 \times 0.16$$

Morphology measurements

Forage morphology measurements of the RGWC and Italian RG lysimeters were taken in mid-September, 2014. Measurements included leaf length, pseudostem length, leaf width, and tiller density. Leaf and pseudostem length were measured using a ruler on the youngest fully unfolded leaf of 10 randomly selected tillers in each lysimeter. Leaf width was measured using digital calipers. Tiller density was recorded by counting the number of tillers in a 0.009 m^2 quadrat at two locations within the lysimeter area.

3.2.8 Soil measurements

Soil sample collection

At the end of the experimental period, once the full mineral nitrogen (NO_3^--N + NH_4^+-N) leachate breakthrough curve had been completed, soil samples were taken from the lysimeters by destructive sampling at 4 depths: 0-15, 15-30, 30-45, and 45-65 cm. These were collected using a hand auger fitted with a bucket auger head (0.08 m diameter) to go down to each depth at four different places in each lysimeter (Plate 3.11), and these were bulked for each lysimeter.

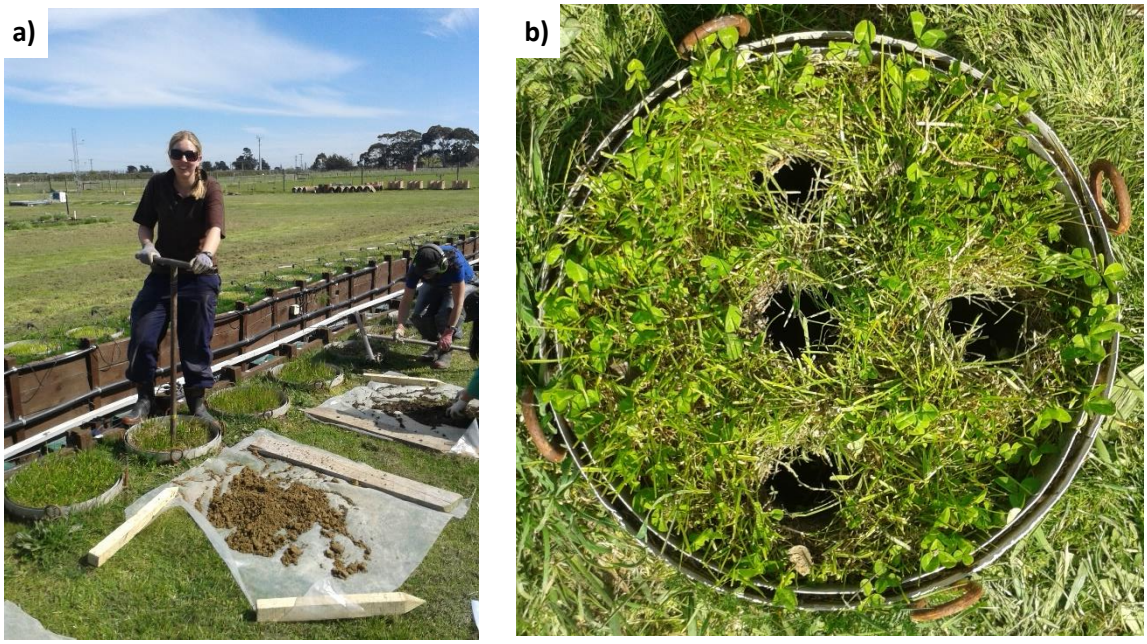


Plate 3.11 Collection of soil samples: a) using hand auger in the field, and b) the arrangement of the four different holes from which the soil samples were bulked.

Analysis of soil

Soil samples were analysed for inorganic N (NH_4^+ and NO_3^-), total N and ^{15}N enrichment. For inorganic N, this involved a KCl extraction, followed by FIA analysis. For the KCl extraction a 5 g sample of field-moist soil was weighed into a 50 mL plastic centrifuge tube containing 25 mL of 2M KCl. This was

shaken for 1 hour (Ratek Platform Mixer, Model: RM2, Victoria, Australia), centrifuged at 4000 rpm for 10 minutes (Heraeus Multifuge 3S-R Centrifuge, Thermo Electron Corporation, Germany) and filtered through Advantec 5C 110 mm filter paper (adapted from Blakemore *et al.*, 1987). Samples were stored in a -20°C freezer prior to being analysed for NO_3^--N and NH_4^+-N concentrations by flow injection analysis using a FOSS FIAstar 5000 twin channel analyser (Foss Tecator AB, Hoganas, Sweden). This is described in more detail in Section 3.2.6.

For total N and ^{15}N enrichment, soil was dried (55°C) and ground to a fine powder using a glass rod. This was then weighed directly into tin capsules and analysed using IRMS (Sercon Ltd, Crewe, CW1 6JT, UK). Soil moisture content was determined by weighing a 10-20 g sample of soil into a paper dish. The sample was then oven dried for 24 hours at 105°C, and re-weighed to give the dry weight. Gravimetric moisture content was determined by the following calculation:

Moisture content (%) = (Moist soil (g) - Dry soil (g))/Dry soil (g) x 100

Soil particle size analysis

The proportions of sand (0.02-2 mm), silt (0.002-0.02 mm), and clay (<0.002 mm) were determined for three lysimeters collected from each field at four depths (0-15, 15-30, 30-45, and 45-65 cm) (Table 3.1). These were analysed at the University of Waikato (Earth Science Department, School of Science, Faculty of Science and Engineering) using a Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK). Samples were prepared by placing half a teaspoon of an air-dried soil sample (sieved at 2 mm) in a beaker with 10% hydrogen peroxide overnight. The next day this was heated gently on a hotplate with an additional 5 mL of hydrogen peroxide. Once the sample had been reduced to approximately 5 mL of slurry it was left to cool, 10 mL of 10% calgon was added, and samples were left overnight. The sample was placed in an ultrasonic bath for 5 minutes before laser diffraction size analysis. This is based on the principle that particles of a given size diffract light through a given angle, which increases with decreasing particle size. A laser is passed through a suspension, and the diffracted light is focused onto a multi-element ring detector. This senses the angular distribution of scattered light intensity. Therefore, a stream of particles can be passed through the beam to generate a stable diffraction pattern (Singer *et al.*, 1988; Konert & Vandenberghe, 1997).

3.2.9 Gas measurements

Gas sample collection

Nitrous oxide fluxes were measured twice a week for the first 3 months of the experiment, and then weekly thereafter up to 7 months. A closed chamber method similar to that described by Hutchinson and Mosier (1981) was used. Chambers were constructed of a metal cylinder, insulated with 2.5 mm thick polystyrene foam to avoid heating of the atmosphere in the chamber during sampling. The headspace was on average 0.14 m above the soil surface. Stainless steel gas rings with an annular

water trough were fitted to each lysimeter before the start of the experiment. Chambers were placed inside these to provide an air-tight seal. Sampling was carried out between 1200 and 1400 hours on each sampling occasion. Headspace samples (20 mL) were collected in 6 mL Exetainers® (Labco Limited, UK) using a syringe inserted through a rubber septum in the top of the chamber. These samples were taken at 0, 20 and 40 minutes after the chambers were placed on top of the lysimeters. Once weekly throughout the measurement period, additional samples were collected for ^{15}N analysis. Following the regular sampling (described above), chambers remained on the lysimeters for a total of 3 hours, after which a larger (40 mL) sample was collected and placed into a 12 mL Exetainer®.

Plate 3.12 Gas sample collection: a) chambers in place over lysimeters, and b) gas sampling apparatus.

Analysis of gas samples

Immediately prior to analysis, gas samples were brought to ambient atmospheric pressure. Concentrations of N_2O were determined using gas chromatography (SRI 8610 gas chromatograph; ^{63}Ni electron capture detector, SRI Instruments, CA, USA) and linked to an autosampler (Gilson 222 XL; Gilson Inc., WI, USA) as described by Clough *et al.* (2010). The gas chromatograph was calibrated using BOC α standards (BOC Scientific, New Zealand) and an air standard was used to check for consistency during each run (air standards obtained from National Institute of Water and Atmospheric Research (NIWA), Wellington). PeakSimple software (SRI Instruments, CA, USA) was used to control and monitor the ECD. Nitrous oxide fluxes were calculated following Hutchinson and Mosier (1981). Cumulative N_2O emissions were determined by integration. Nitrous oxide ^{15}N enrichment was determined by IRMS (Sercon Ltd, Crewe, CW1 6JT, UK). Gas samples were prepared using a TGII trace gas system, equipped with cryo-trapping and focusing, to isolate the species of interest.

3.2.10 ^{15}Nitrogen isotope analysis

The ^{15}N enrichment of the diffused samples was analysed on a continuous flow IRMS (Sercon Ltd, Crewe, CW1 6JT, UK). Solid samples, such as herbage and soil (dried and ground), were initially combusted at 1000°C in an oxygen atmosphere in an automated Dumas-style elemental analyser which was linked to the 20-22 stable isotope ratio mass spectrometer.

3.2.11 ^{15}N balance calculations

Percentage ^{15}N recoveries in leachate, herbage, soil and N_2O emissions were calculated using the equation from Cabrera and Kissel (1989):

$$\%^{15}N\ recovery = 100 \times \frac{\mathrm{p(c-b)}}{\mathrm{f(a-b)}}$$

where:

% ^{15}N recovery = ^{15}N in measured fraction as a percentage of the ^{15}N applied

p = moles of N in the sample

c = atom% ^{15}N enrichment of the sample (from IRMS analysis)

b = atom% ^{15}N in Control (non-urine treated) fraction

f = moles of N in the urine applied to the lysimeter (0.975 mol)

a = atom% ^{15}N enrichment of the urine-N applied to the lysimeter (4.884%)

Leachate

The N leaching loss (g) for each lysimeter was calculated by summing the NH_4^+ and NO_3^- and then multiplying this concentration (mg N L^{-1}) by the drainage volume on each sampling occasion, and dividing by 1000. This was then used to determine the total moles of N leached from each lysimeter by dividing the total mass of N leached from each lysimeter by the molar mass of N (14.0067 g mol^{-1}). Interpolation was used to estimate the atom% ^{15}N enrichment for those sampling occasions where ^{15}N analyses were not performed. Nitrogen ^{15}N recovery was determined using the equation above for each leaching event, then these were summed to give the total ^{15}N recovery in leachate during the experimental period.

Herbage

For each lysimeter, the mass of N (g) in herbage at each harvest was determined by multiplying the dry matter harvested (g) by the N content (%) in the herbage, measured by the Elementar Vario-Max CN Elemental Analyser (Elementar GmbH, Hanau, Germany) and dividing by 100. The moles of N at each harvest were then determined by dividing the mass of N by the molar mass of N (14.0067 g mol^{-1}). Herbage samples from six of the 14 harvests were analysed by IRMS so interpolation was used to estimate the ^{15}N enrichment for those sampling occasions where ^{15}N analyses were not performed.

Nitrogen ^{15}N recovery was determined using the equation above for each harvest, then these were summed to determine the total ^{15}N recovery in herbage during the experimental period.

Soil

The total mass of soil at each depth (0-15 cm, 15-30 cm, 30-45 cm, and 45-65 cm) was determined using the following equation:

$$M_{soil} = \frac{V_s \times \mathrm{P_b} \times \mathrm{C}}{1000}$$

where:

M_{soil} = mass (kg) of dry soil in each depth

V_s = volume of soil in each depth (m^3)

P_b = dry bulk density of soil in each soil depth (g cm^{-3})

C = conversion factor for cm^3 to m^3 (1,000,000 cm^3 = 1 m^3)

Soil bulk density (g cm^{-3}) was measured for all lysimeters at the 0-15 cm depth using cores (0.054 m diam., 0.05 m deep) which were carefully pressed into the soil, then oven-dried for 48 hours at 105°C. However, for the other depths, soil bulk density was determined from cores taken in triplicate from the field where the lysimeters were collected. The mass of N (g) in each soil depth was determined by multiplying the total N content (mg kg^{-1}) in the soil at each depth by the mass of the soil (M_{soil}) and dividing by 1000 to convert mg to g. This was then used to determine the moles by dividing the mass of N (g) by the molar mass of N (14.0067 g mol^{-1}). Nitrogen ^{15}N recovery was determined for each depth, then these were summed to calculate the total ^{15}N recovery in the soil of each lysimeter at the end of the experimental period.

Nitrous oxide emissions

The moles of N evolved as N_2O (moles N_2O-N $lysimeter^{-1}$ d^{-1}) were determined from the N_2O flux (mg N m^{-2} h^{-1}), by first converting to units of g N $lysimeter^{-1}$ d^{-1} and then dividing by the molar mass of N (14.0067 g mol^{-1}). Nitrogen ^{15}N enrichment was determined for four N_2O sampling dates, so interpolation was used to estimate the N_2O-^{15}N enrichment for those sampling occasions where ^{15}N analyses were not performed. Nitrogen ^{15}N recovery was determined for each sampling date, then integration was used to determine the total recovery of N_2O-^{15}N emitted during the measurement period.

Errors

The 95% confidence intervals were calculated to indicate the error associated with the ^{15}N recovery for each N fraction measured using the equation:

$$Confidence\ interval\ (95\%) = \frac{1.96 \times s}{\sqrt{n}}$$

where:

s = standard deviation

n = sample size (number of replicates)

This was also calculated for the total N pool measured for each treatment combination by summing the variances of each N fraction, plus twice the covariance of all the possible two-way combinations of these fractions (Legg & Meisinger, 1982):

$$Var(N_T) = Var(N_1) + Var(N_2) + Var(N_3) + Var(N_4) + 2Cov(N_1, N_2) + 2Cov(N_1, N_3) + 2Cov(N_1, N_4) + 2Cov(N_2, N_3) + 2Cov(N_2, N_4) + 2Cov(N_3, N_4)$$

where:

$Var(N_T)$ = the total variance for the measured N pool

Var = the variance for each N fraction

N_1 to N_4= the four different N fractions

Cov = the covariance for the two-way combinations of the fractions (determined using Minitab 17, Minitab Inc.)

3.2.12 Statistical analysis

Data were analysed by conducting an analysis of variance (ANOVA) for a split-plot design using Genstat (18th Edition, VSN International Ltd.). Control data were excluded for N leaching loss analysis of variance because the values were very low (<2.2 kg N ha^{-1}), as expected, and the treatments of interest were forage type and GA application. Where significant effects were shown, the unrestricted LSD procedure (Saville, 1990) at either the 5% or 10% level was used to identify differences among means. Soil data were analysed for each soil depth, while for forage quality data, season means were analysed.

3.3 Results

3.3.1 Climate conditions and water inputs

During the experimental period (7 May 2014 to 1 October 2015), the average daily air temperature ranged from a low of 0°C in July 2015 to a high of 23°C in February 2015 (Figure 3.4a). Similarly, daily average soil temperature (10 cm depth) ranged from 2°C (July 2015) to 24°C (January 2015) (Figure 3.4a). Temperatures followed expected cyclical trends with warmer temperatures during summer and cooler temperatures during winter. For 3 weeks following the application of GA, soil temperatures ranged from 6.7°C to 12.4°C, this was within the recommended soil temperature range of 5°C-16°C for GA application on pasture (Matthew *et al.*, 2009). Water inputs for the 17-month experimental period totalled 1965 mm, comprising 713 mm of rainfall, and 1252 mm of irrigation or simulated rainfall (Figure 3.4b). The winter of 2014 was drier than average so the majority of the water inputs during this season were simulated rainfall, rather than actual rain.

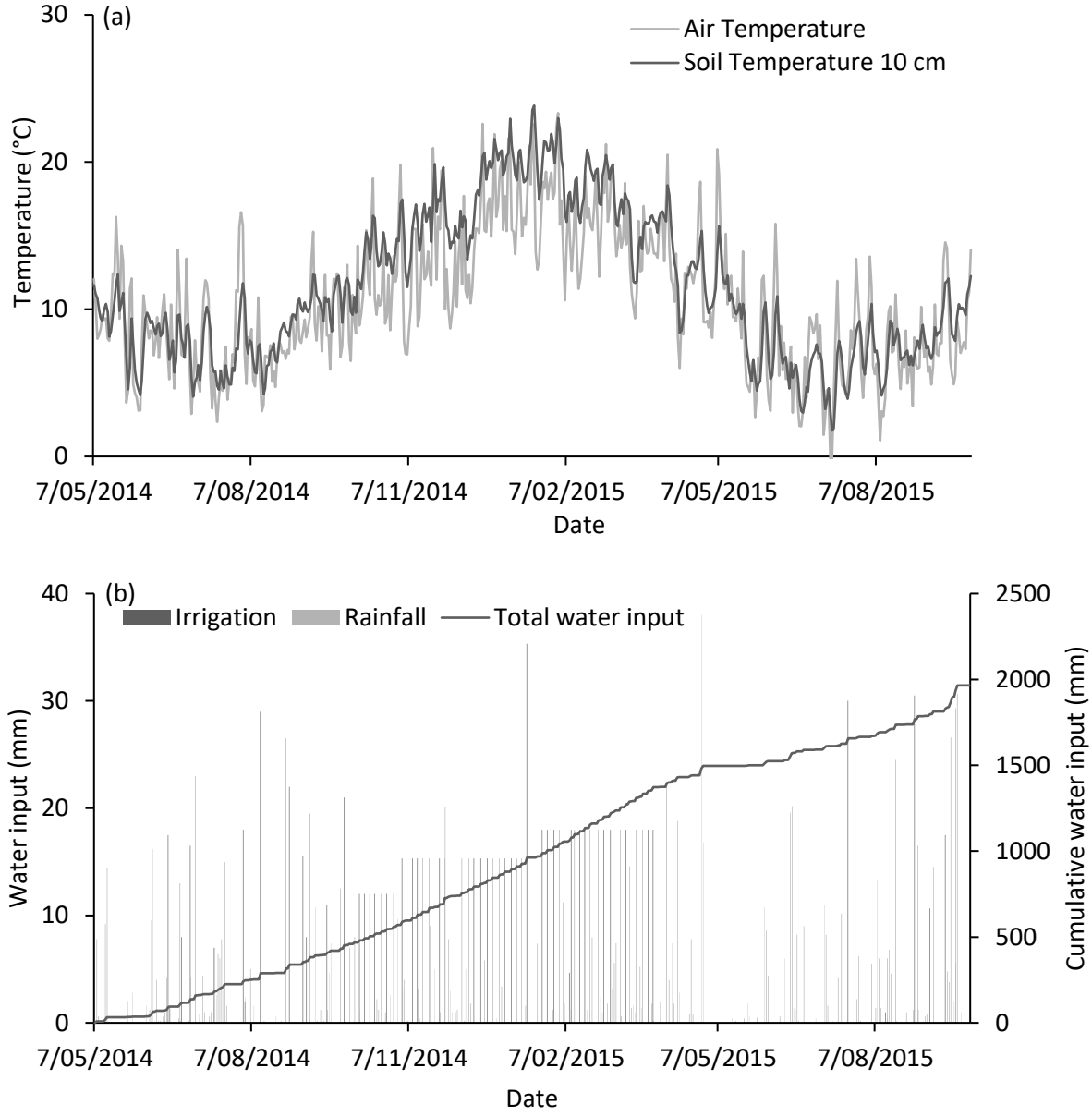
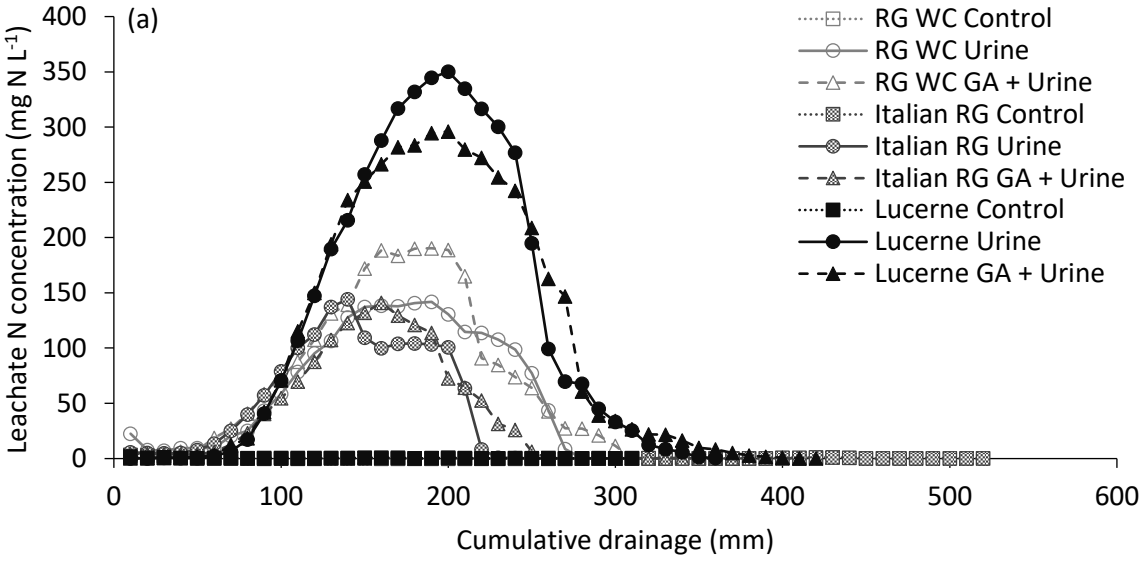


Figure 3.4 a) Average daily air temperature and soil temperature (at 10 cm), and b) daily and cumulative rainfall and irrigation (including simulated rainfall) water inputs for the experimental period: 7 May 2014 to 1 October 2015.

3.3.2 Nitrogen leaching losses

A breakthrough curve of the leachate mineral N concentrations (NO_3^--N + NH_4^+-N) shows that the concentrations increased with drainage to a peak and then declined to background levels (Figure 3.5a). Peak concentration values ranged from 141 to 350 mg N L^{-1} for urine-treated lysimeters (Figure 3.5a). Total mineral N leaching losses (NO_3^--N + NH_4^+-N) were 35.3% lower ($P < 0.1$) from Italian RG (132.6 kg N ha^{-1}) and 98.5% higher ($P < 0.001$) from lucerne (407.2 kg N ha^{-1}), when compared with RGWC (205.1 kg N ha^{-1}) (Figure 3.5b). The application of GA did not significantly affect N leaching losses ($P = 0.469$). Similarly, there was no interaction ($P = 0.185$) between forage type and treatment (Urine, GA + Urine). Leaching losses from Control lysimeters were minimal (<2.2 kg N ha^{-1}). There was an exceptionally low total N leaching loss for one replicate of the RGWC-GA + Urine treatment; if this value were to be excluded from the statistical analysis, the revised mean value for this treatment is 245.3 kg N ha^{-1}, so the GA effect is significant for RGWC ($P < 0.05$). Also RGWC and Italian RG would differ overall ($P < 0.05$), and the forage type x treatment interaction would be significant ($P < 0.05$). Total drainage volumes ranged from 321 mm (lucerne-Control) to 502 mm (Italian RG-Control) over the experimental period. There was no difference in total drainage due to forage type ($P = 0.217$), however, treatment ($P < 0.05$) and the forage x treatment ($P < 0.05$) interactions were both significant due to the high drainage from the Italian RG-Control lysimeters.



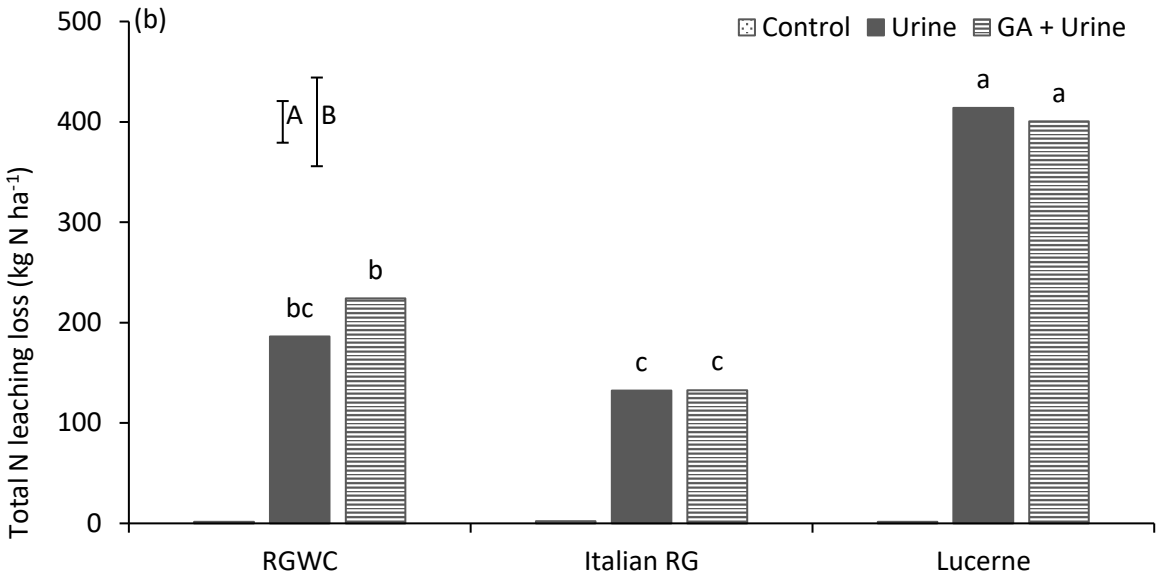


Figure 3.5 Mean mineral nitrogen leaching loss (NO_3^--N + NH_4^+-N): a) concentration (mg N L^{-1}) in leachate plotted against cumulative drainage, and b) total mineral N leaching loss (kg N ha^{-1}) from lysimeters for the experimental period: 7 May 2014 to 1 October 2015. Perennial ryegrass and white clover (RGWC), Italian ryegrass (Italian RG) and lucerne were treated in May 2014 with either: Control, Urine, or GA + Urine (gibberellic acid (GA) at 8 g GA ha^{-1}, urine at 700 kg N ha^{-1}). Control means are plotted but not included in the statistical analysis. The error bars are least significant differences (LSD) at the 5% level; LSD A is for comparing two means for a particular forage type, and LSD B is for all other comparisons. Bars with the same letter (a-c) are not significantly different at the 5% level.

3.3.3 Herbage yield and nitrogen uptake

Total herbage yield (t DM ha^{-1}) (Figure 3.6a) and N uptake (kg N ha^{-1}) (Figure 3.6b) harvested over the 17-month experimental period were both affected by forage type and treatment (Control, Urine, GA + Urine) with a significant forage type x treatment interaction. For RGWC and Italian RG, herbage yield and N uptake were higher ($P < 0.05$) for both the urine-treated lysimeters when compared with the respective controls (Figure 3.6a, b). The herbage yield and N uptake for the Italian RG-Control were particularly low ($P < 0.001$) at only 12.2 t DM ha^{-1} and 246 kg N ha^{-1}, respectively, compared with the range of 20 to 25.7 t DM ha^{-1} and 629 to 872 kg N ha^{-1} for all other treatments. The application of GA had no significant effect on total herbage yield or N uptake of any of the forage types during the experimental period. Lucerne showed no difference in herbage yield or N uptake across all treatments (Control, Urine, GA + Urine).

Winter N uptake (kg N ha^{-1} d^{-1}) for urine-treated forage (Urine and GA + Urine) was greatest ($P < 0.001$) for the Italian RG at 2.13 kg N ha^{-1} d^{-1} on average, compared with 1.56 kg N ha^{-1} d^{-1} for RGWC and 0.25 kg N ha^{-1} d^{-1} for lucerne across the two harvests which occurred in winter 2014 (Table 3.4).

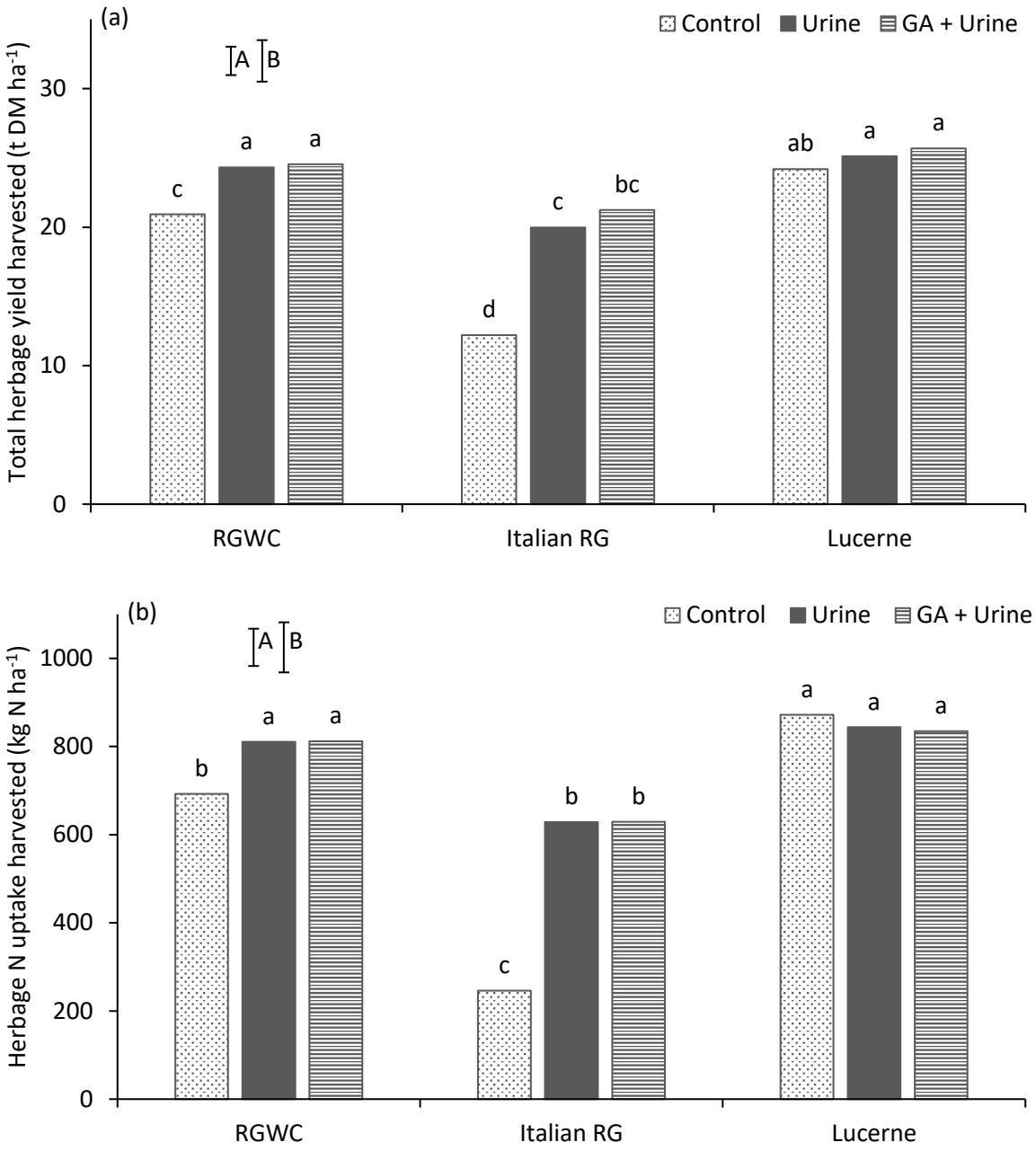


Figure 3.6 Herbage: a) total dry matter yield harvested (t DM ha^{-1}), and b) nitrogen uptake harvested (kg N ha^{-1}) from lysimeters for the experimental period: 7 May 2014 to 1 October 2015. Perennial ryegrass and white clover (RGWC), Italian ryegrass (Italian RG) and lucerne were treated in May 2014 with either: Control, Urine, or GA + Urine (gibberellic acid (GA) at 8 g GA ha^{-1}, urine at 700 kg N ha^{-1}). The error bars are least significant differences (LSD) at the 5% level; LSD A is for comparing two means for a particular forage type, and LSD B is for all other comparisons. Bars with the same letter (a-d) are not significantly different at the 5% level.

Table 3.4 Nitrogen uptake harvested (kg N ha^{-1} d^{-1}) for winter herbage (DM yield x N% ÷ rotation length), perennial ryegrass-white clover (RGWC), Italian ryegrass (Italian RG), and lucerne. Treatments of: Control (no urine, water), Urine (700 kg N ha^{-1}), GA + Urine (GA applied at 8 g GA ha^{-1}, with 700 kg N ha^{-1} urine) were applied in May 2014.

Forage Type	Treatment	Harvest 23/06/2014 (kg N ha^{-1} d^{-1})	Harvest 7/08/2014 (kg N ha^{-1} d^{-1})	Mean winter N uptake (kg N ha^{-1} d^{-1})
RGWC	Control	0.72	0.50	0.62[c]
RGWC	Urine	1.60	1.62	1.61[b]
RGWC	GA[1] + Urine	1.67	1.32	1.51[b]
Italian RG	Control	0.22	0.10	0.16[d]
Italian RG	Urine	1.91	2.30	2.10[a]
Italian RG	GA + Urine	2.02	2.30	2.16[a]
Lucerne	Control	0.34	-[2]	0.18[d]
Lucerne	Urine	0.48	-[2]	0.25[d]
Lucerne	GA + Urine	0.48	-[2]	0.25[d]
P value	Forage type			<0.001
P value	Treatment			<0.001
P value	FxT			<0.001
LSD A[3]				0.1549
LSD B				0.1660

[1]Gibberellic acid; [2]Lucerne was not harvested on 7/08/2014 due to insufficient plant growth and following seasonal management guidelines (Moot *et al.*, 2003). Means with the same letter are not significantly different at the 5% level. [3]LSD A is for comparing two means for a particular forage type, and LSD B is for all other comparisons.

3.3.4 ^{15}N balance

The total recovery of the ^{15}N applied ranged from 78.5% to 85.5% (±5.5 and 2.9% confidence interval (95%), respectively). Recovery of ^{15}N within the herbage, leachate, and N_2O N pools was affected by forage type ($P < 0.001$), but the soil-N pool was unaffected (Table 3.5). Herbage-^{15}N recoveries were highest for Italian RG (47.8-49.5%), and lowest for lucerne (16.4-18.4%) ($P < 0.05$), compared with 36.3% to 40.1% for RGWC (Table 3.5, Figure 3.7). Leachate-^{15}N recovery was greater ($P < 0.05$) from lucerne (50.5-52.5%) compared with RGWC and Italian RG, which had recoveries of 23.7-27.9% and 16.8%, respectively (Table 3.5, Figure 3.8). There were no significant differences in ^{15}N recovery for the soil fraction, with values ranging from 13 to 17.6% (Table 3.5). The recovery of ^{15}N as gaseous N_2O emissions was much lower than the other fractions. The highest N_2O-^{15}N recoveries were from the Italian RG and the RGWC-Urine treatments (0.9-1.0%), and the lowest from the lucerne (0.4-0.5%) ($P < 0.05$) (Table 3.5). There were no differences ($P > 0.05$) in the ^{15}N recovered in the herbage, leachate, or soil between the Urine and GA + Urine treatments, however, for the N_2O emissions there was a reduction ($P < 0.05$) in the ^{15}N recovered as N_2O from the RGWC forage when GA was applied,

compared with the urine only treatment. As this was only observed for one forage type, the overall effect of GA on ^{15}N recovery was minimal.

Herbage-N derived from urine varied between forage type with the N in Italian RG having the greatest amount derived from urine-N (56.1-58.1%), RGWC had the second highest at 33-36.8%, and 14.5-16% of N in lucerne herbage was derived from urine (Table 3.6). Of the total N leached 84.7-86.3% of this was derived from the applied urine, which demonstrates that only around 15% was derived from soil-N or fertiliser-N (Table 3.6). At the end of the experimental period, only 1-1.3% of the total soil-N was derived from the urine-^{15}N applied (Table 3.6). Of the N_2O emissions measured, 45.9-66.6% was derived from the urine-^{15}N (Table 3.6).

Herbage-^{15}N recovery at each harvest date is plotted against time in Figure 3.7. For the first three harvests Italian RG had the highest ^{15}N recovery at 11.1-12.5% (47-131 days), then steadily decreased. The next highest ^{15}N recovery was in the RGWC herbage, where values ranged from 4.9% to 8.1% for the first five harvests (47-182 days) before also declining. Lucerne herbage had the lowest ^{15}N recovery over this time with 1.7% recovered at the first harvest, recovery then peaked at 4.7-5.5% on days 131 and 156, and then declined.

The recovery of ^{15}N in the soil was most influenced by soil depth, the greatest recovery occurred in the 0-15 cm depth with values ranging from 8.1% to 12.6% (Figure 3.9). The RGWC and Italian RG-Urine had the highest soil-^{15}N recoveries in this top layer ($P < 0.05$). The 15-30 cm layer had much lower ^{15}N recoveries of 2-3.8%, with the highest being for lucerne ($P < 0.05$). For the 30-45 cm and 45-65 cm depths these recoveries were 1-1.8% and 0.6-1.7%, respectively.

Table 3.5 Recovery (%) of the ^{15}N applied with the urine, in the herbage, leachate, soil, and N_2O fractions (n = 5). Numbers in each column with the same letter (a-c) are not significantly different at the 5% level.

Forage Type	Treatment	Herbage (total)	Leachate (total)	Soil (total)	N_2O emissions	TOTAL	Unaccounted for
RGWC	Urine	40.1 [b] ± 3.0[1]	23.7 [b] ± 9.1	16.7 ± 2.0	1.0 [a] ± 0.1	81.5 ± 8.8	18.5
	GA + Urine	36.3 [b] ± 6.4	27.9 [b] ± 12.4	17.6 ± 3.6	0.7 [b] ± 0.2	82.5 ± 3.9	17.5
Italian RG	Urine	49.5 [a] ± 4.8	16.8 [b] ± 5.4	16.4 ± 3.2	0.9 [a] ± 0.2	83.6 ± 3.3	16.4
	GA + Urine	47.8 [a] ± 3.4	16.8 [b] ± 4.1	13.0 ± 3.8	0.9 [a] ± 0.1	78.5 ± 5.5	21.5
Lucerne	Urine	18.4 [c] ± 5.2	52.5 [a] ± 8.2	14.1 ± 2.6	0.5 [c] ± 0.1	85.5 ± 2.9	14.5
	GA + Urine	16.4 [c] ± 4.6	50.5 [a] ± 4.7	14.3 ± 1.5	0.4 [c] ± 0.1	81.6 ± 3.1	18.4
***P* Value[2]**	**Forage**	***	***	NS	***	NS	
	Treatment	NS	NS	NS	NS	NS	
	FxT	NS	NS	NS	NS	NS	
	LSD A[3]	5.31	4.96	4.02	0.20	4.95	
	LSD B	5.71	11.51	4.33	0.21	8.19	

[1]± 95% Confidence interval;

[2]NS nonsignificant, ***Significant at the 0.001 probability level;

[3]LSD A is the 5% LSD for comparing two means for a particular forage type, and LSD B is for all other comparisons

Table 3.6 Percentage (%) of the N in herbage, leachate, soil, and N_2O emissions which was derived from the applied urine (n = 5). Numbers in each column with the same letter (a-c) are not significantly different at the 5% level.

Forage Type	Treatment	Herbage (total)	Leachate (total)	Soil (total)	N_2O emissions
RGWC	Urine	36.8 [b] ± 3.8[1]	85.0 ± 8.6	1.3 [a] ± 0.0	61.0 [a] ± 2.5
	GA + Urine	33.0 [b] ± 5.8	84.7 ± 1.7	1.1 [bc] ± 0.2	51.2 [b] ± 9.7
Italian RG	Urine	58.1 [a] ± 3.4	86.1 ± 3.2	1.3 [ab] ± 0.2	66.6 [a] ± 3.8
	GA + Urine	56.1 [a] ± 1.8	85.8 ± 2.1	1.1 [bc] ± 0.2	64.6 [a] ± 5.1
Lucerne	Urine	16.0 [c] ± 4.2	86.2 ± 5.5	1.0 [c] ± 0.1	49.9 [b] ± 3.6
	GA + Urine	14.5 [c] ± 3.9	86.3 ± 2.8	1.0 [c] ± 0.1	45.9 [b] ± 6.4
P Value[2]	Forage	***	NS	NS	***
	Treatment	NS	NS	*	NS
	FxT	NS	NS	NS	NS
	LSD A[3]	4.83	6.67	0.23	9.24
	LSD B	5.49	6.93	0.21	8.77

[1]± 95% Confidence interval;

[2]NS nonsignificant, *Significant at the 0.05 probability level, ***Significant at the 0.001 probability level;

[3]LSD A is the 5% LSD for comparing two means for a particular forage type, and LSD B is for all other comparisons

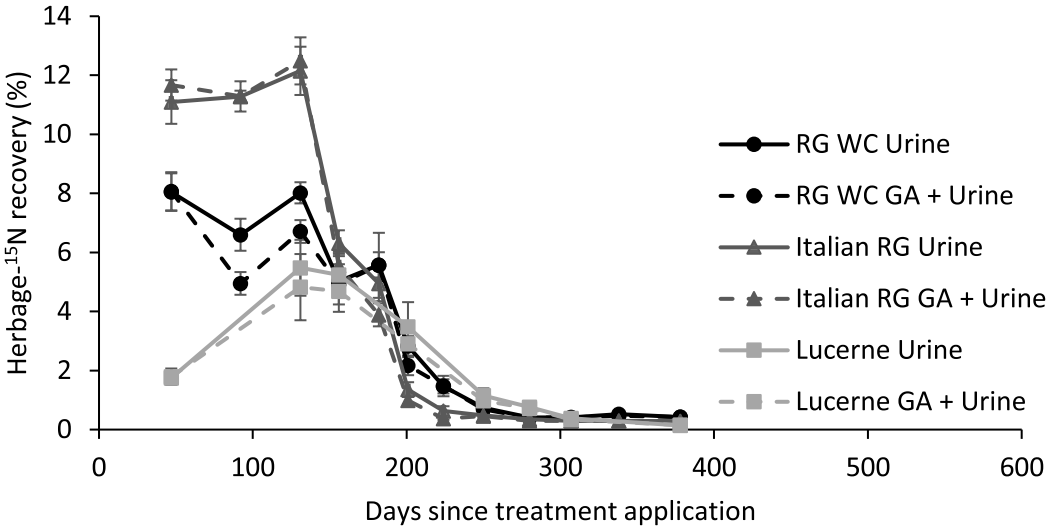


Figure 3.7 Herbage-^{15}N recovery (%) throughout the experimental period: 7 May 2014 to 1 October 2015. Perennial ryegrass and white clover (RGWC), Italian ryegrass (Italian RG) and lucerne were treated in May 2014 with either: Control, Urine, or GA + Urine (gibberellic acid (GA) at 8 g GA ha^{-1}, urine at 700 kg N ha^{-1}). Error bars are standard error of the mean (n = 5).

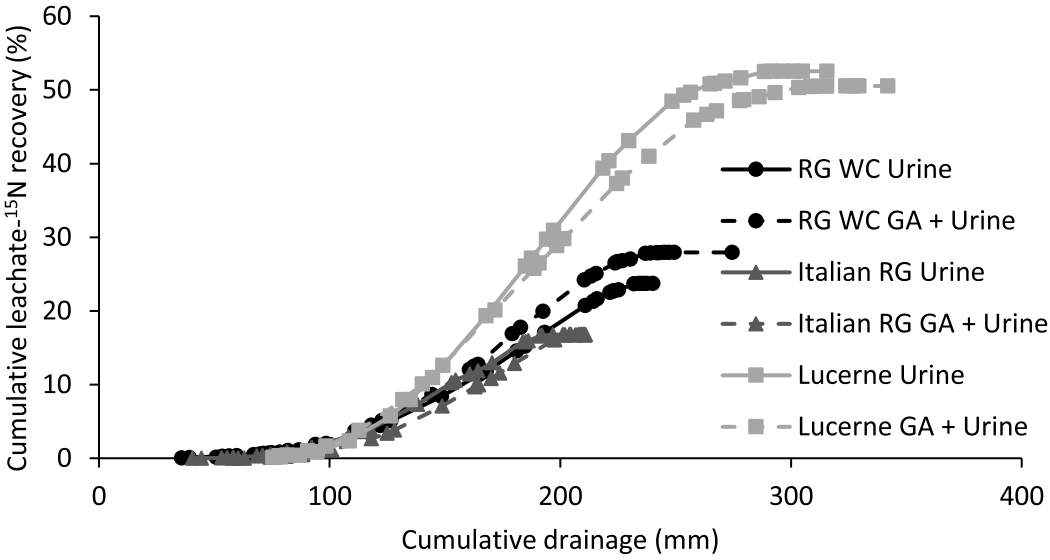


Figure 3.8 Mean cumulative recovery of leachate-^{15}N (%) against drainage over the experimental period: 7 May 2014 to 1 October 2015. Perennial ryegrass and white clover (RGWC), Italian ryegrass (Italian RG) and lucerne were treated in May 2014 with either: Control, Urine, or GA + Urine (gibberellic acid (GA) at 8 g GA ha^{-1}, urine at 700 kg N ha^{-1}).

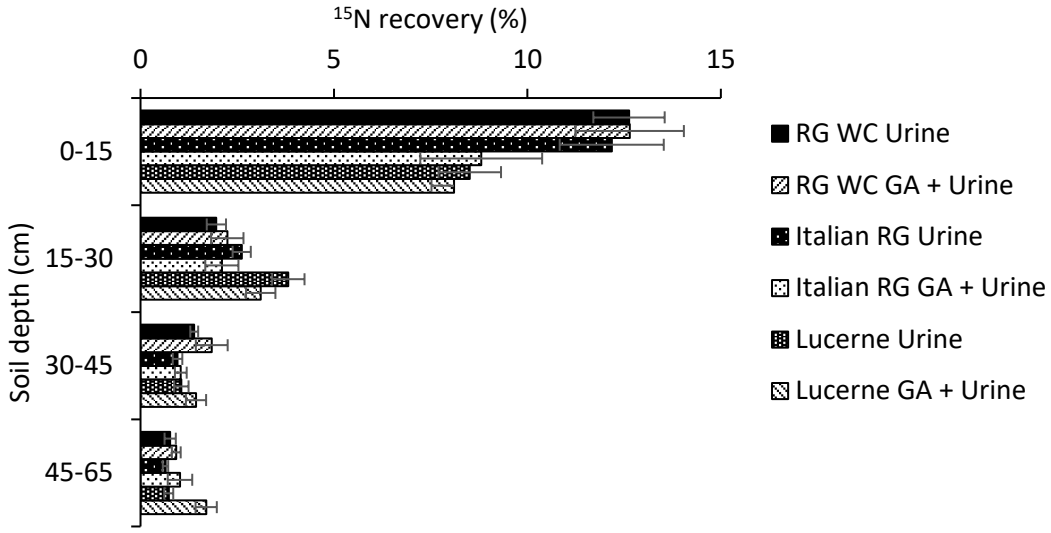


Figure 3.9 Soil-^{15}N recovery (%) at each soil depth: 0-15, 15-30, 30-45, 45-65 cm at the end of the experimental period. Perennial ryegrass and white clover (RGWC), Italian ryegrass (Italian RG) and lucerne were treated in May 2014 with either: Control, Urine, or GA + Urine (gibberellic acid (GA) at 8 g GA ha^{-1}, urine at 700 kg N ha^{-1}). Error bars are standard error of the mean (n = 5).

3.3.5 Forage quality

Seasonal means for forage quality parameters: water soluble carbohydrates (WSC), crude protein (CP), and metabolisable energy (ME) are shown in Tables 3.7-3.9. Detailed data for each harvest are presented in Appendix C, Table C 3 and Figure C 1. Water soluble carbohydrates varied through time and tended to be highest in summer. When looking at the main effects for each season, Italian RG herbage had the highest levels of WSC (152-393 mg g^{-1}) throughout the experimental period. The RGWC herbage had the second highest WSC levels (112-329 mg g^{-1}), and lucerne had the lowest WSC levels in winter, summer and autumn (89-184 mg g^{-1}) (Table 3.7). Across all seasons, there was no significant difference in herbage WSC levels between the Urine, and the GA + Urine treatments. However, in the winter and spring following treatment application, WSC levels in herbage were higher for Control lysimeters, than those which had received urine applications (Urine, GA + Urine). This was lower than the urine-treated lysimeters in summer, and not significantly different in autumn (Table 3.7).

Table 3.7 Mean seasonal water soluble carbohydrate (mg g^{-1}) levels in herbage throughout the experimental period: 7 May 2014 to 1 October 2015. Perennial ryegrass and white clover (RGWC), Italian ryegrass (Italian RG) and lucerne were treated in May 2014 with either: Control, Urine, or GA + Urine (gibberellic acid (GA) at 8 g GA ha^{-1}, urine at 700 kg N ha^{-1}).

		Winter	Spring	Summer	Autumn
Forage					
	RG WC	112 [b]	202 [b]	329 [b]	137 [b]
	Italian RG	152 [a]	286 [a]	393 [a]	172 [a]
	Lucerne	89 [c]	184 [b]	169 [c]	96 [c]
	P-value	<0.001	<0.001	<0.001	<0.001
	LSD (5%)	14.3	21.5	32.4	16.0
Treatment					
	Control	167 [a]	243 [a]	280 [b]	137 [a]
	Urine	92 [b]	211 [b]	304 [a]	128 [a]
	GA + Urine	95 [b]	218 [b]	308 [a]	140 [a]
	P-value	<0.001	<0.001	0.011	0.157
	LSD (5%)	8.3	15.9	19.3	13.0
Forage	**Treatment**				
RG WC	**Control**	142 [b]	198 [de]	290 [c]	140 [bc]
RG WC	**Urine**	91 [cd]	213 [cd]	347 [b]	128 [c]
RG WC	**GA + Urine**	105 [c]	195 [de]	350 [b]	142 [bc]
Italian RG	**Control**	261 [a]	362 [a]	377 [ab]	170 [a]
Italian RG	**Urine**	95 [cd]	236 [bc]	397 [a]	163 [ab]
Italian RG	**GA + Urine**	99 [c]	262 [b]	404 [a]	182 [a]
Lucerne	**Control**	99 [c]	170 [e]	171 [d]	102 [d]
Lucerne	**Urine**	89 [cd]	183 [de]	167 [d]	92 [d]
Lucerne	**GA + Urine**	81 [d]	198 [d]	169 [d]	95 [d]
P-value	FxT	<0.001	<0.001	0.074	0.744
	LSD A	14.4	27.6	33.3	22.6
	LSD B	17.5	29.4	39.9	23.1

Crude protein levels were highest in the lucerne herbage throughout the experimental period when looking at the main effects (Table 3.8). The RGWC herbage had the second highest CP levels in all seasons except spring where it was not significantly different from lucerne. Italian RG herbage had the lowest CP throughout the experimental period. There was no significant difference between the Urine, and the GA + Urine treatments in winter, spring, or summer, and in the autumn (a year after treatment application) herbage treated with GA + Urine had lower CP levels ($P < 0.05$) with a mean across the forages of 203 mg g^{-1}, compared with 210-212 mg g^{-1} for the other treatments (Control, Urine). In the winter and spring following treatment application, the CP in the herbage of the urine-treated lysimeters (Urine, GA + Urine) was significantly higher than that of the Control treatment. In contrast, in summer the Control lysimeters had the highest ($P < 0.05$) CP levels. In autumn, there was no significant difference between the Control and the Urine treatments (Table 3.8).

Table 3.8 Mean seasonal crude protein (mg g^{-1}) levels in herbage throughout the experimental period: 7 May 2014 to 1 October 2015. Perennial ryegrass and white clover (RGWC), Italian ryegrass (Italian RG) and lucerne were treated in May 2014 with either: Control, Urine, or GA + Urine (gibberellic acid (GA) at 8 g GA ha^{-1}, urine at 700 kg N ha^{-1}).

		Winter	Spring	Summer	Autumn
Forage					
	RG WC	275 [b]	218 [a]	180 [b]	223 [b]
	Italian RG	256 [c]	174 [b]	109 [c]	164 [c]
	Lucerne	321 [a]	227 [a]	201 [a]	238 [a]
	P-value	<0.001	<0.001	<0.001	<0.001
	LSD (5%)	4.0	11.0	13.7	13.6
Treatment					
	Control	241 [b]	200 [b]	178 [a]	212 [a]
	Urine	307 [a]	210 [a]	157 [b]	210 [a]
	GA + Urine	304 [a]	208 [a]	154 [b]	203 [b]
	P-value	<0.001	0.024	<0.001	0.021
	LSD (5%)	7.3	7.7	10.3	6.7
Forage	**Treatment**				
RG WC	**Control**	249 [d]	226 [b]	202 [ab]	225 [bc]
RG WC	**Urine**	292 [bc]	206 [c]	168 [c]	230 [b]
RG WC	**GA + Urine**	285 [c]	221 [b]	171 [c]	214 [c]
Italian RG	**Control**	175 [e]	131 [e]	119 [d]	166 [d]
Italian RG	**Urine**	297 [b]	202 [c]	107 [de]	165 [d]
Italian RG	**GA + Urine**	296 [b]	189 [d]	101 [e]	161 [d]
Lucerne	**Control**	300 [b]	242 [a]	214 [a]	246 [a]
Lucerne	**Urine**	332 [a]	223 [b]	197 [ab]	234 [b]
Lucerne	**GA + Urine**	331 [a]	214 [bc]	191 [b]	233 [b]
P-value	FxT	<0.001	<0.001	0.489	0.177
	LSD A	12.6	13.3	17.9	11.6
	LSD B	10.8	14.6	18.9	15.7

Metabolisable energy was highest for both RGWC and Italian RG in winter following treatment application when looking at the main effects. In spring, ME was highest in the Italian RG, whereas, in summer and autumn, RGWC had the highest ME. Lucerne had the lowest ME in all seasons except summer, when Italian RG had the lowest ME (Table 3.9). There was no significant difference in herbage ME between Urine, and GA + Urine treatments across all seasons. The application of urine (Urine, GA + Urine) significantly reduced the ME of the herbage in the winter and spring following treatment application. In summer, there was no significant effect of urine treatment on ME of herbage, and in autumn the ME of the Control was significantly higher than that of the Urine treatment, but the GA + Urine was not significantly different from either of them.

Table 3.9 Mean seasonal metabolisable energy (MJ kg^{-1} DM) of herbage throughout the experimental period: 7 May 2014 to 1 October 2015. Perennial ryegrass and white clover (RGWC), Italian ryegrass (Italian RG) and lucerne were treated in May 2014 with either: Control, Urine, or GA + Urine (gibberellic acid (GA) at 8 g GA ha^{-1}, urine at 700 kg N ha^{-1}).

		Winter	Spring	Summer	Autumn
Forage					
	RG WC	12.0 ^{a}	11.8 ^{b}	11.6 ^{a}	11.9 ^{a}
	Italian RG	12.1 ^{a}	11.9 ^{a}	10.3 ^{c}	11.6 ^{b}
	Lucerne	11.6 ^{b}	10.6 ^{c}	10.6 ^{b}	10.8 ^{c}
	P-value	<0.001	<0.001	<0.001	<0.001
	LSD (5%)	0.16	0.09	0.14	0.13
Treatment					
	Control	12.1 ^{a}	11.6 ^{a}	10.9 ^{a}	11.5 ^{a}
	Urine	11.8 ^{b}	11.4 ^{b}	10.8 ^{a}	11.4 ^{b}
	GA + Urine	11.8 ^{b}	11.3 ^{b}	10.8 ^{a}	11.4 ^{ab}
	P-value	<0.001	<0.001	0.155	0.024
	LSD (5%)	0.12	0.08	0.15	0.10
Forage	**Treatment**				
RG WC	**Control**	12.1 ^{b}	11.8 ^{b}	11.6 ^{a}	12.0 ^{a}
RG WC	**Urine**	11.9 ^{bc}	11.7 ^{b}	11.6 ^{a}	11.8 ^{ab}
RG WC	**GA + Urine**	11.9 ^{bc}	11.7 ^{b}	11.6 ^{a}	11.9 ^{a}
Italian RG	**Control**	12.6 ^{a}	12.2 ^{a}	10.4 ^{cd}	11.6 ^{c}
Italian RG	**Urine**	11.9 ^{c}	11.7 ^{b}	10.3 ^{d}	11.5 ^{c}
Italian RG	**GA + Urine**	11.9 ^{c}	11.8 ^{b}	10.2 ^{d}	11.7 ^{bc}
Lucerne	**Control**	11.6 ^{d}	10.7 ^{c}	10.8 ^{b}	11.0 ^{d}
Lucerne	**Urine**	11.6 ^{d}	10.6 ^{c}	10.6 ^{bc}	10.8 ^{e}
Lucerne	**GA + Urine**	11.6 ^{d}	10.3 ^{d}	10.5 ^{cd}	10.8 ^{e}
P-value	FxT	<0.001	<0.001	0.294	0.373
	LSD A	0.21	0.14	0.26	0.18
	LSD B	0.22	0.14	0.24	0.18

3.3.6 Herbage morphology and botanical composition

Morphology of the RGWC and Italian RG are shown in Figure 3.10 for September 2014. Leaf length, pseudostem length, leaf width, and tiller density were all affected by forage type and treatment (Control, Urine, GA + Urine) and all had a significant forage type x treatment interaction. Leaf and pseudostem length were both not significantly different across treatments for the RGWC forage type. However, for urine-treated (Urine, GA + Urine) Italian RG herbage these were higher (values of 38 and 12 cm, respectively), compared with the RGWC (~26 and 7.5 cm, respectively) ($P < 0.05$). The Italian RG Control was shown to have the lowest leaf and pseudostem lengths ($P < 0.05$) (Figure 3.10a, b). Leaf width was highest for the Italian RG forage type, the urine-treated Italian RG with the widest leaves (7 to 7.4 mm), and Italian RG Control the next widest ($P < 0.05$) (Figure 3.10c). The RGWC-Urine lysimeters had the lowest leaf width at 3.6 mm but the highest tiller density at 11433 tillers m^{-2}

($P < 0.05$) (Figure 3.10c, d). There were no significant differences in tiller density between the other treatments (433-6756 tillers m^{-2}) (Figure 3.10d).

Botanical composition data for RGWC show that the Control lysimeters had a higher ($P < 0.05$) proportion of white clover (and therefore the lowest proportion of perennial ryegrass), compared with those which were urine-treated (Urine, GA + Urine) (Figure 3.11). Although the GA + Urine treated lysimeters had slightly higher proportions of white clover than the Urine only treatment, this was not significantly different.

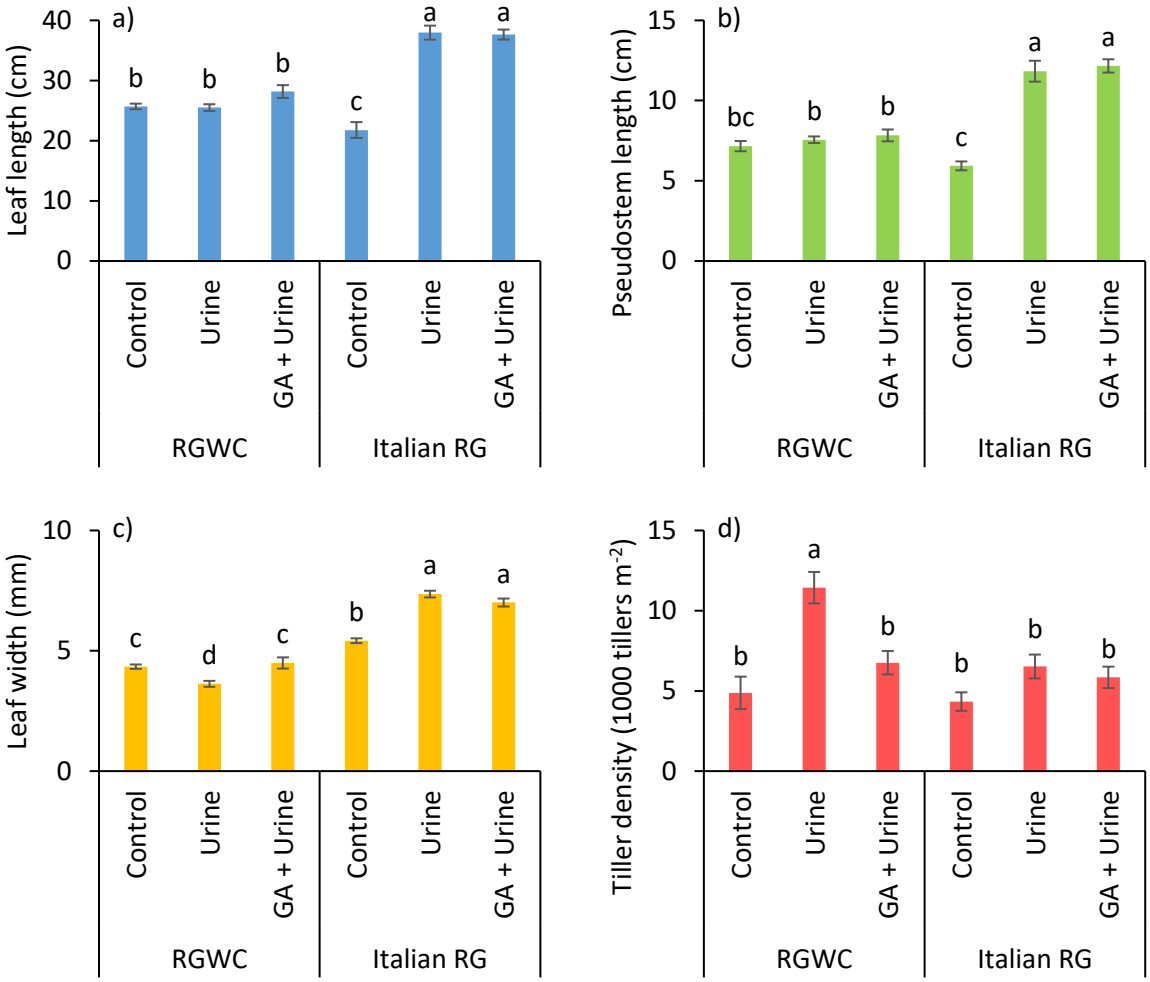


Figure 3.10 Herbage morphology: a) leaf length, b) pseudostem length, c) leaf width, and d) tiller density for the perennial ryegrass-white clover (RGWC) and Italian ryegrass (Italian RG) measured 15-24 September 2014. Treatments of: Control (no urine, water), Urine (700 kg N ha^{-1}), GA + Urine (GA applied at 8 g GA ha^{-1}, with 700 kg N ha^{-1} urine) were applied in May 2014. Bars with the same letter are not significantly different at the 5% level.

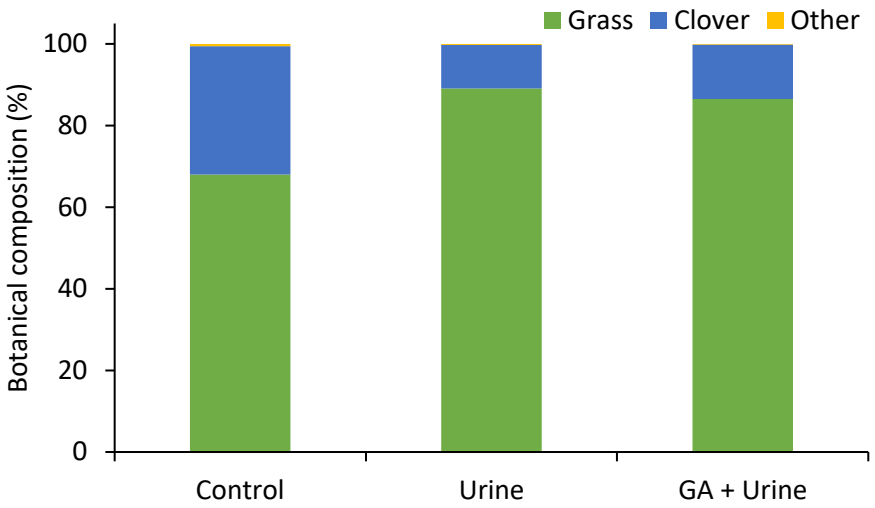


Figure 3.11 Botanical composition of perennial ryegrass-white clover (RGWC) on 15 September 2014, 131 days after treatment application. Treatments of: Control (no urine, water), Urine (700 kg N ha^{-1}), GA + Urine (GA applied at 8 g GA ha^{-1}, with 700 kg N ha^{-1} urine) were applied in May 2014.

3.3.7 Soil

Soil NH_4^+-N concentrations (mg NH_4^+-N kg $soil^{-1}$) were not significantly different at the 0-15 cm depth and had values of 0.025-0.059 mg NH_4^+-N kg $soil^{-1}$ (Figure 3.12a). However, soil NO_3^--N concentrations at this depth (mg NO_3^--N kg $soil^{-1}$) were affected by forage type and were highest for lucerne at 0.115 mg NO_3^--N kg $soil^{-1}$, compared with 0.053 and 0.023 mg NO_3^--N kg $soil^{-1}$ for RGWC and Italian RG, respectively (Figure 3.12b). At the 15-30 cm depth, soil NH_4^+-N concentrations were influenced by forage type and were highest for lucerne at 0.045 mg NH_4^+-N kg $soil^{-1}$, compared with 0.01-0.015 mg NH_4^+-N kg $soil^{-1}$ for the other forages. Soil NO_3^- at this depth was affected by forage type (P = 0.020), and treatment (P = 0.026) and was again higher for lucerne (0.025 mg NO_3^--N kg $soil^{-1}$), than the other forage types (0.007-0.010 mg NO_3^--N kg $soil^{-1}$). Nitrate concentrations for the different treatments decreased in the order of GA + Urine > Urine > Control. At the 30-45 cm and 45-65 cm soil depths, NH_4^+ and NO_3^- concentrations in the soil were not significantly different, and NO_3^- levels had decreased to below detection limits.

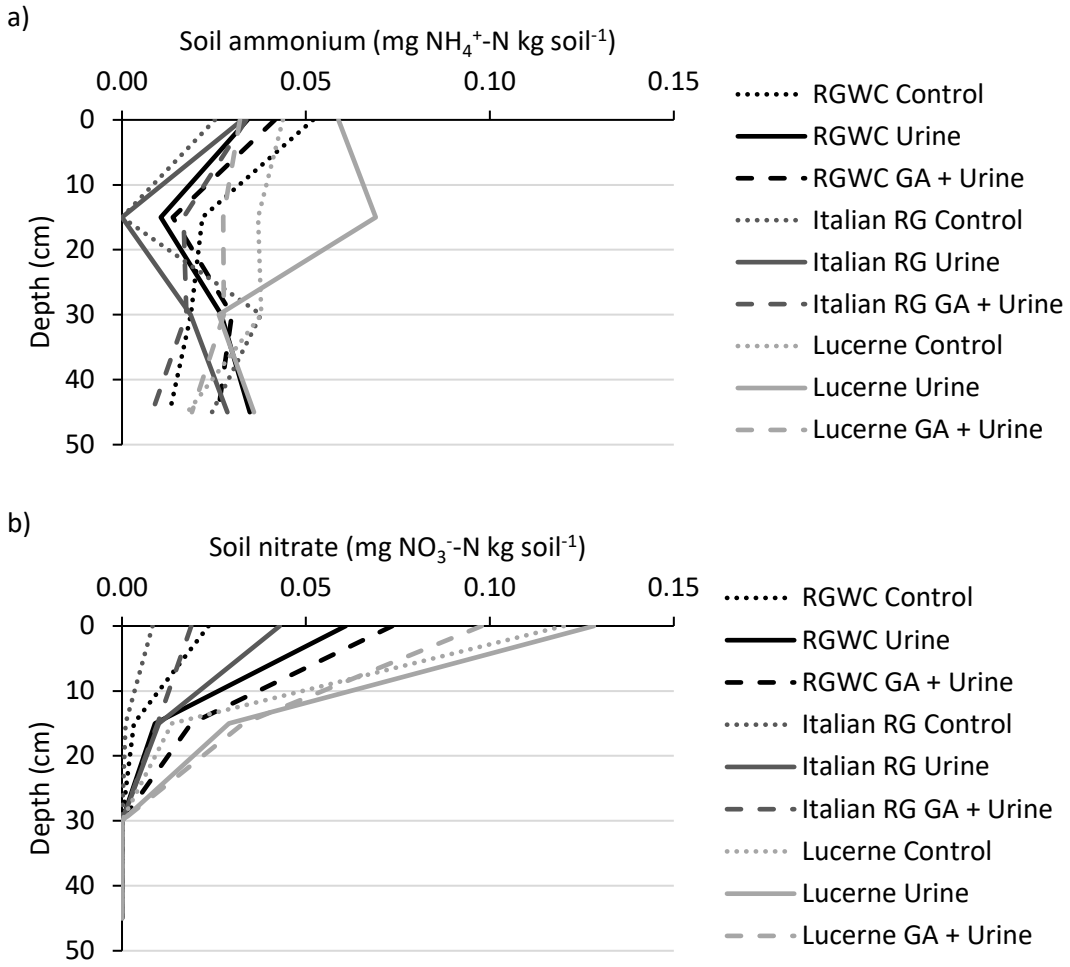


Figure 3.12 Soil: a) ammonium, and b) nitrate concentrations (mg N kg $soil^{-1}$) at the end of the 17-month experimental period. Perennial ryegrass and white clover (RGWC), Italian ryegrass (Italian RG) and lucerne were treated in May 2014 with either: Control, Urine, or GA + Urine (gibberellic acid (GA) at 8 g GA ha^{-1}, urine at 700 kg N ha^{-1}).

Control lysimeters were not analysed for total N. Total N in the soil decreased with depth and was affected by forage type (P = 0.005) and there was a significant forage type x treatment interaction (P = 0.027) at the 0-15 cm depth. Total N was highest for RGWC-GA + Urine, Lucerne-GA + Urine, Lucerne-Urine at 0.22-0.25%, compared with the other lysimeter treatments 0.17-0.19% (Figure 3.13). At the 15-30 cm depth there was a significant forage type x treatment interaction (P = 0.023). The RGWC-GA + Urine and lucerne-Urine had the highest values (0.16-0.17%). At the 30-45 cm depth, forage type (P = 0.003), treatment (P = 0.002), and forage type x treatment (P < 0.001) had an effect on total N. The RGWC-GA + Urine had the highest total N at 0.10%, compared with 0.05-0.06% for the others. There were no significant differences in total N for the 45-65 cm soil depth.

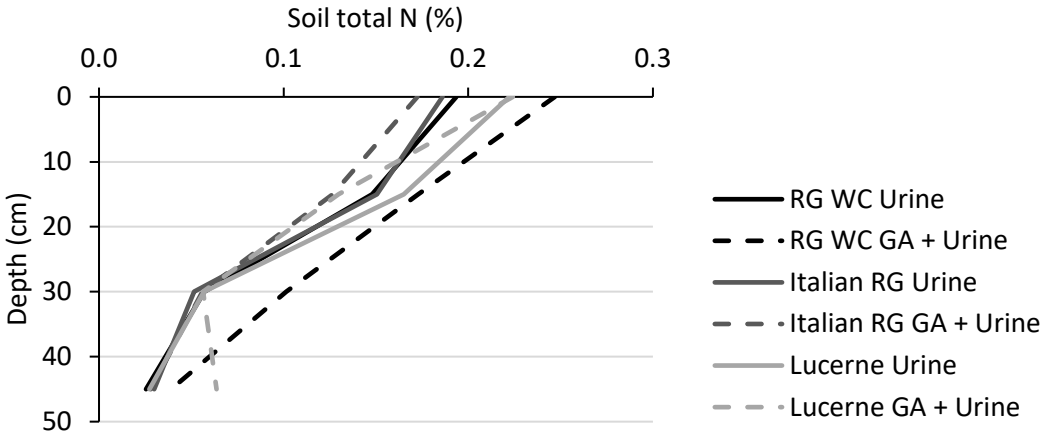


Figure 3.13 Soil total N (%) at the end of the 17-month experimental period. Perennial ryegrass and white clover (RGWC), Italian ryegrass (Italian RG) and lucerne were treated in May 2014 with either: Control, Urine, or GA + Urine (gibberellic acid (GA) at 8 g GA ha^{-1}, urine at 700 kg N ha^{-1}).

3.4 Discussion

3.4.1 Italian ryegrass

The reduced leaching loss from the Italian RG is in agreement with Malcolm *et al.* (2014) who attributed a 24-54% lower leaching loss from Italian ryegrass, compared with other forage species, to higher plant winter activity (which included plant growth and root metabolic activity). In a more detailed study of root architecture comparing Italian ryegrass and tall fescue (*Festuca arundinacea* Schreb.), Malcolm *et al.* (2015) showed that plant growth was more important than root architecture for recovery of N during winter. Although in the current study, total herbage yields for urine-treated forage during the experimental period of 17 months were 16% lower for Italian RG than for RGWC (Figure 3.6a), the N uptake during the winter months (June-August 2014) was 37.3% higher for Italian RG than RGWC (Table 3.4). This indicates that Italian RG grew more during the cool winter period than RGWC and was able to take up more N in this period, supporting the findings of Malcolm *et al.* (2014) and Malcolm *et al.* (2015). Other studies have also shown Italian ryegrass to have lower N leaching losses than perennial ryegrass when treated with urine at rates of 600-700 kg N ha^{-1} (Popay & Crush, 2010; Moir *et al.*, 2013). Similarly, following a 300 kg N ha^{-1} application of $K^{15}NO_3$, drainage volumes and NO_3^- concentrations in leachate from hybrid/Italian ryegrass cultivars were lower, and uptake of ^{15}N was higher, compared with perennial ryegrass (Nichols & Crush, 2007). In an earlier study, Italian ryegrass was also shown to have the second highest ^{15}N recovery of the species tested and observations indicated that Italian ryegrass has the ability to grow roots deeper than 1 m (Crush *et al.*, 2005).

3.4.2 Lucerne

The high leaching loss from lucerne in this experiment was unexpected because many previous studies have described the ability of lucerne to take up water (Brown *et al.*, 2005; Moot *et al.*, 2008) and N from depth (Mills & Moot, 2010; Black & Moot, 2013). Similarly Betteridge *et al.* (2007) measured NO_3^- leaching losses from a lucerne crop harvested for silage/hay (with no direct grazing or urine deposition) using ceramic suction cups (0.6 m deep). Leaching losses from the lucerne crop were 10-24 kg NO_3^--N ha^{-1} y^{-1} which was the same or slightly more than the ryegrass-red clover-white clover mixture in their experiment. However, a lucerne crop harvested for silage/hay would not contain urine patches and thus the potential leaching loss would be expected to be lower than if the lucerne was grazed (i.e. as simulated in this current PhD study). Similarly, N loading in urine patches may be expected to be higher in lucerne than RGWC due to the higher crude protein intake with lucerne. In related work, Smith (2015) noted that the urine-N concentration was 24 to 77% higher for cows grazing irrigated lucerne than irrigated perennial ryegrass-white clover.

The main reason for the high N leaching loss under lucerne was the low winter herbage growth rate and N uptake. Table 3.4 shows winter N uptake for lucerne treated with urine to be very low, 84% less than RGWC. From June to August, lucerne growth was minimal while temperatures were cool and so it was not harvested in August, when RGWC and Italian RG were harvested. This is typical of lucerne and is a recommended management practice (Moot *et al.*, 2003). Although McKenzie *et al.* (1990) found most lucerne roots to be in the top 0.2 m of the soil, some roots were found down to 0.9 m. Other studies have reported the presence of lucerne roots as deep as 6 m (Mathers *et al.*, 1975) and 10 m (Forde *et al.*, 1989). This suggests a limitation in the lysimeter measurement technique for lucerne in the current study because the lysimeters used were only 0.7 m deep. For deep soils, N leached below 0.7 m may possibly still be captured by the lucerne plant roots when spring growth commences. However, the soil-^{15}N data do not really support this with such small amounts of urine-N found in the soil below 15 cm. The current experiment was irrigated as this is typical management practice for dairy farms in the Canterbury area. However, due to its water use efficiency and deep rooting ability, lucerne is often also used as a dryland crop. For an on-farm situation, where no irrigation is applied, leaching losses from lucerne would likely be lower than the current experiment because the soil would be drier than irrigated land (thus requiring a greater volume of water to wet the soil and create drainage). In the second winter the lucerne lysimeters took much longer to wet up and drain and this suggests that it is possible for these plants to dry out the soil so much over the spring-early autumn period that this could possibly reduce subsequent winter drainage. More research is required in dryland lucerne systems.

3.4.3 Gibberellic acid

Gibberellic acid application to a 700 kg N ha^{-1} urine patch had no effect on N leaching loss, herbage DM yield, herbage N uptake, or forage quality parameters such as WSC, and calculated ME. The lack of effect on DM yield is in contrast with earlier work, where forage treated with lower rates of N (20-50 kg N ha^{-1}), applied as fertiliser, has been shown to provide an additive DM response to an application of GA (Morgan & Mees, 1958; van Rossum *et al.*, 2013; Ghani *et al.*, 2014; Zaman *et al.*, 2014). Ghani *et al.* (2014) and Zaman *et al.* (2014) also found that CP content was decreased by GA application. van Rossum *et al.* (2013) found this effect in a range of forages except perennial ryegrass-white clover and attributed the effect of GA on CP to changes in clover content in the forages. It is likely that the high rate of urine-N (700 kg N ha^{-1}) used in the current study overrode the effect of the GA. The effect of GA application on DM yield, N uptake, and N leaching loss across a range of lower rates of urine-N is later discussed in Chapter 6. In the current experiment, GA application had no effect on CP of herbage in winter, spring and summer, but in autumn (a year after treatment application) the herbage treated with GA had a significantly lower CP. Similarly, a botanical composition carried out in September of the first year showed a slight but nonsignificant increase in clover content with GA application, compared with the urine only treatment. Morgan and Mees (1958) showed that GA caused a 1.5-2% decrease in N content, although CP yields followed a similar trend to DM yield and increases with GA and N were additive. Other reductions in N and CP content have also been reported for forage species (Scurfield, 1958; McGrath & Murphy, 1976; Percival, 1980) and Champeroux (1962) showed GA improved N utilisation through increased DM yield but decreased N uptake and N content. In their first experiment Biddiscombe *et al.* (1962) found no significant difference in total yield of N over all 5 harvests.

In contrast to these studies, Parsons *et al.* (2013) found a major increase in DM production in winter-derived plants at both low and high N, with no evidence of a reduction of N content of tissues. This suggested that the extra growth increased N uptake from the soil environment. They suggested that this offers prospects for reducing environmental impacts (leaching, N_2O) compared with obtaining the same yield increase by adding fertiliser-N. Similarly, Morgan and Mees (1958) described increased uptake of N with GA at the first harvest, though decreases of CP at harvest 2 were often observed. Some studies have also noted increases in total N or CP yield due to DM increases with GA (Morgan & Mees, 1958; Finn & Nielsen, 1959; Biddiscombe *et al.*, 1962). There have been many studies carried out using other plant species, which could still have some relevance to pastoral plants. Gibberellic acid application was shown to increase N in *Calendula officinalis* L. (Mohamed & Ebtsam, 2013), linseed (Khan *et al.*, 2010), and in wheat (Brian *et al.*, 1954). A study by Livné and Vaadia (1965) showed transpiration rate of barley increased following GA application, this indicates GA could increase water

use by plants which could potentially reduce N leaching, though this was not measured in their experiment.

As described by Whitehead and Edwards (2015) the potential impact of GA on N leaching does not just relate to the N uptake of the forages. An increase in DM, as shown in many of the experiments in the literature (but not in the current study over the 17-month experimental period) could reduce the use of nitrogen fertilisers, and therefore reduce the inputs of N cycling through that farm system. However, it is important to note that this may be offset by an increase in legume content following GA application which would add more N to the system through fixation and could increase the CP of the diet of grazing animals. Whitehead and Edwards (2015) estimated that one application of GA would result in a relative reduction in N_2O emission per urination of 18% when compared with those when using N fertiliser. It is important to measure forage quality parameters in studies looking at the effects of GA, this is because a reduction in forage CP, caused by the GA application could lower the N excretion of grazing animals (Whitehead & Edwards, 2015). This was observed in autumn in the current experiment, and in some of these earlier experiments in the literature. Despite the current experiment having indicated that at the urinary-N rate of 700 kg N ha^{-1}, an autumn application of GA would not reduce N leaching loss, increase DM yield or N uptake, it is possible that at lower rates of urinary-N a response to GA may occur (this is further investigated in Chapter 6 later in the thesis).

3.4.4 ^{15}N balance

The higher recovery of urine-^{15}N in leachate and lower ^{15}N recovery in herbage from the lucerne treatment demonstrates that lucerne was not as active in taking up N during the drainage period. Though not significant at the 5% level, the Italian RG tended to have a lower proportion (17%) of the applied ^{15}N in the leachate, compared with RGWC (24-28%). This reinforces the findings of the leaching loss data, which show a 35.3% reduction in leaching losses from Italian RG, compared with RGWC. Italian RG herbage-^{15}N recoveries (47.8-49.5%) were higher than that found by Malcolm *et al.* (2015) who reported a 30.4% recovery, but lower than Sorensen and Jensen (1996) who reported 61.2-69.3%. Both of these earlier studies used lower rates of urine-N, 300 kg N ha^{-1} and 205 kg N ha^{-1}, respectively. The higher herbage recovery reported by Sorensen and Jensen (1996) could be attributed to the urine in their study being spring-applied, whereas Malcolm *et al.* (2015) and the current study used autumn-applied urine.

These findings indicate that Italian ryegrass is efficient at taking up urinary-N deposited by grazing animals both in the autumn and spring. It was also the most efficient of the three forage types in the current study. The ^{15}N recovery of 36-40% for the RGWC herbage is consistent with the findings of other studies (McLaren *et al.*, 1993; Di *et al.*, 2002; Silva *et al.*, 2005; Buckthought, 2013). At cold root

temperatures (<14°C) Italian and perennial ryegrasses have both been shown to preferentially take up NH_4^+ under conditions where pH, external NH_4^+/NO_3^- concentration, plant N status, and pretreatment root temperature were varied (Clarkson & Alison, 1979). Therefore, the preferred form of N uptake is unlikely to be the cause of the difference in winter N uptake of perennial ryegrass and Italian ryegrass. Instead, this is likely to relate to winter activity/growth and is reinforced by the results in Figure 3.7 which show Italian RG herbage recovered higher amounts of urine-^{15}N during the winter through to early spring in the first year (June-September, harvests 47, 91, and 131 days after treatment application), compared with the RGWC and lucerne forages.

Consistent with previous ruminant urine studies, N_2O-^{15}N recoveries were ≤1% and made up a very low proportion of the total N balance. However, it is an important environmental concern and should not be ignored as pollution swapping could be of concern. For example, if a reduction in N leaching occurs, but causes a subsequent increase in N_2O emissions it would not be considered to be reducing the environmental impact of the agricultural system. The N_2O emission data (daily flux, total emissions, and emission factors (EF_3) for the experimental period) were previously reported (Di *et al.*, 2016) and showed no differences between the Italian RG and RGWC forages, however, emissions from lucerne were lower ($P < 0.05$). Di *et al.* (2016) suggested that this was due to the high amount of urine-N leached from the lucerne lysimeters. The ^{15}N leaching data confirm this. The current study reinforces these findings as only 0.38-0.46% of urine-N was recovered as N_2O from the lucerne, compared with 0.7-0.97% for the RGWC and Italian RG. Overall the ^{15}N recovery as N_2O in the current study fitted within the 0.015 to 2.2% range of values found in the literature (Table 2.1). The ^{15}N recovery as N_2O is typical of previous results (Table 2.1). The results for RGWC were similar to those found by Clough *et al.* (1998) who applied a 1000 kg N ha^{-1} urine patch in winter to perennial ryegrass-white clover forage and recovered 1% of the applied ^{15}N as N_2O on a sandy loam soil. These values, however, were higher than those found by Selbie (2014) who reported a N_2O-^{15}N recovery of 0.48% for a 1000 kg N ha^{-1} urine patch applied in winter to perennial ryegrass, and by Buckthought (2013) who recovered 0.57% from an 800 kg N ha^{-1} urine patch applied in autumn to perennial ryegrass-white clover. These values were more similar to the lucerne recovery in the current study.

Not all of the ^{15}N applied was recovered in the current study, with total ^{15}N recovery ranging from 78.5 ±5.5% to 85.5 ±2.9%. The 14.5-21.5% of the applied ^{15}N which was not recovered was likely lost via ammonia volatilisation, denitrification to N_2, or removed within plant roots and stubble, none of which were measured. This was similar, or slightly less than the amount of ^{15}N that is typically unaccounted for in ^{15}N balance studies (Fraser *et al.*, 1994; Clough *et al.*, 1998; Decau *et al.*, 2003; Welten *et al.*, 2013; Selbie, 2014; Buckthought *et al.*, 2015).

The majority (84.7-86.3%) of the N lost as leachate was derived from the urine-N applied, however, for herbage-N this varied considerably with forage type. It is possible that the lower urine-N contributing to the herbage N yield for RGWC and lucerne forages was due to the presence of the N-fixing species: white clover (RGWC) and lucerne fixing atmospheric N_2. Nitrogen fixation rates for white clover and lucerne have been reported to be 13-342 and 51-319 kg N $ha^{-1}y^{-1}$, respectively (Ledgard & Steele, 1992), while an earlier review by Evans and Barber (1977) reported values of 104-160 and 128-600 kg N ha^{-1} y^{-1}, respectively.

The influence of soil depth on urine-^{15}N recovery in the soil, shown in Figure 3.9, has been previously reported in other studies. For example Di *et al.* (2002) observed 30-40% of the urine-^{15}N in the top 5 cm of the soil and that this decreased with soil depth. In agreement with the current study, they also found significant amounts of urine-N remained in the soil profile 1 year after the urine application. They suggested that as most of this soil-N was present in the roots or soil organic matter, it would be released gradually by decomposition or mineralisation in subsequent years. Fraser *et al.* (1994) also found 79% of the urinary-^{15}N recovered in soil was in the top 20 cm soil depth. They suggested that this could have been immobilised and that some of the soil-N on the surface may have previously been taken up by the herbage, but now returned to the soil following death of pasture (e.g. root turnover). Similarly, Selbie (2014) showed that of the urinary ^{15}N recovered in soil, 69% was found in the top 15 cm of the soil, and again this decreased with depth. The majority of the ^{15}N measured in the soil was in organic forms which indicated immobilisation processes had occurred. Because the findings of the current study align with these other studies, it is likely that the majority of the soil-^{15}N which remained in the soil at the end of the 17-month experimental period was also mostly in organic forms (including microbial biomass) as a result of immobilisation and the mechanisms described above, though these were not measured in the current study. Even studies which used different sources of N inputs e.g. pig slurry (Carey *et al.*, 1997) and urea fertiliser (Prasertsak *et al.*, 2001) at much lower rates, had similar trends in ^{15}N recovered in the soil, where the majority was in the top 10 cm, and decreased with depth.

To the author's knowledge, this study is the first of its kind to attempt to determine the effect of GA application on the fate of urine-N in grazed forage systems. The results showed that the application of GA to urine on the three forages had no effect on the herbage uptake of urine-^{15}N. This was unexpected because as previously mentioned a study by Parsons *et al.* (2013) showed a major increase in forage yield of winter-derived plants treated with GA at both low and high N levels, and there was no evidence of a reduction of tissue N content. They suggested that this extra growth had increased the uptake of N from the soil environment. Therefore, it is reasonable to have expected to see an increase in herbage urine-^{15}N uptake in the current study, however this was not observed. Other studies have indicated that application of GA to forage can increase the N yield (kg N ha^{-1}) (Morgan & Mees, 1958; Finn &

Nielsen, 1959; Biddiscombe *et al.*, 1962), but often reduces the N content (%) in the forage (Finn & Nielsen, 1959; Percival, 1980; Ghani *et al.*, 2014). Based on the results of the current study, the application of GA is not recommended as a mitigation tool to directly reduce urine-N losses, however, further studies could investigate the effect of urine-N rate, and timing of application to determine if there is an effect at lower levels of N (see Chapter 6 for effect of urine-N rate).

3.5 Conclusions

- Nitrogen leaching losses were 35.3% lower from Italian RG than RGWC. This was attributed to the Italian RG having higher winter activity and the ability to take up more N during the cool winter period than RGWC. The high leaching losses from lucerne were attributed to poor winter herbage growth of the lucerne and the limitation of the 0.7 m deep lysimeters for N leaching measurements of this deep rooting species. For these reasons, Italian ryegrass rather than lucerne is recommended as an alternative to perennial ryegrass-white clover in terms of reducing N leaching losses.

- The application of GA to a 700 kg N ha^{-1} urine patch had no effect on N leaching loss, herbage DM yield or herbage N uptake. Similarly, GA had little effect on the recovery of urinary-^{15}N, compared with when urine was applied alone. At this stage GA is not recommended as a mitigation tool for minimising N leaching loss or increasing the herbage uptake of urine-N.

- The ^{15}N isotope data showed Italian ryegrass was the most efficient of the three forages for utilising urine-N deposited in autumn, recovering more urine-^{15}N and reducing the proportion of urine-N leached over the subsequent winter. Lucerne, was shown to have a lower N use efficiency with the highest amounts of urine-^{15}N leached over winter. Despite the limitations of this study in measuring leaching losses from lucerne, this study has shown that winter-active forages (such as Italian ryegrass) are more effective at taking up autumn-applied urine-N than the other plants tested.

- The environmental benefits of Italian ryegrass warrant investigation into other alternative forage species worldwide. This research is relevant to grazed systems around the world and the greater N use efficiency could also be of relevance to other farming systems.

Chapter 4

Microbiology Pot Experiment

4.1 Introduction

In New Zealand agricultural systems, animals predominantly graze forages outdoors year-round. Most of the N in the urine deposited by these grazing animals is present as urea, that is rapidly hydrolysed to NH_4^+ in the soil. This is then converted to NO_3^- by the process of nitrification. Nitrification is an important biogeochemical process and is associated with many pathways in which N is lost from soils. The NO_3^- which is formed by nitrification is highly susceptible to loss via leaching or denitrification. The oxidation of ammonia is the first rate-limiting step of the nitrification process and is performed, in soil, by the ammonia monooxygenase (AMO) enzyme associated with ammonia-oxidising bacteria (AOB) and ammonia-oxidising archaea (AOA) (Cameron *et al.*, 2013). A previous local study showed nitrate leaching was significantly related to the abundance of the AOB population but not to the AOA population (Di *et al.*, 2009a) and the abundance of AOB accounted for 51% of the variation in nitrate leaching loss.

Chapter 3 showed that lysimeters planted with Italian ryegrass (*Lolium multiflorum* Lam.) (Italian RG) leached 35% less N than those planted with standard perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) (RGWC). The research results showed that Italian RG was more efficient at taking up urinary-N during the winter period, when cool conditions limit plant growth, than RGWC. However, it was unclear whether this was solely responsible for the reduction in N leaching. The current experiment aims to improve our understanding of the mechanisms involved in this observation.

It is possible that under the Italian RG, conditions were less suitable for nitrification of the urine-derived NH_4^+. Factors affecting nitrification include: soil texture, soil structure, temperature, moisture, aeration, pH, electrical conductivity, C:N ratio, cation exchange capacity, and organic matter (Subbarao *et al.*, 2006b). Many of these factors are likely to have been the same for both the Italian RG and RGWC lysimeters in Chapter 3 as they were collected from the same site and soil type. However, the Italian RG could have influenced some of the conditions (e.g. soil moisture, pH, C:N ratio) throughout the experimental period. It was not possible to take samples of the soil in the lysimeters throughout the duration of Lysimeter Experiment 1 (Chapter 3) without disturbing the soil profile. Soil samples were only taken at the end of the 17-month experimental period (Sections 3.2.8, and 3.3.7).

Another mechanism by which Italian RG may have reduced NO_3^- leaching in Chapter 3 could be through the release of a biological nitrification inhibitor (BNI). Some plants release root exudates capable of inhibiting the microbes involved in the soil nitrification process (Bremner & McCarty, 1993; Fillery, 2007; Zakir *et al.*, 2008; Gopalakrishnan *et al.*, 2009; Subbarao *et al.*, 2009; Nardi *et al.*, 2013; Moreta *et al.*, 2014). By retaining N in the form of NH_4^+ for longer, plants have more time to absorb the soil-N, and the NO_3^- pool available for leaching is reduced (Subbarao *et al.*, 2012). Italian ryegrass (*Lolium perenne* L. ssp. *Multiflorum* (Lam.) Husnot (Italian ryegrass cv. Nioudaichi)) has previously been shown to have some BNI activity (Subbarao *et al.*, 2007) but it is unclear if this was the case in Lysimeter Experiment 1 (Chapter 3)

Therefore, the objectives of this experiment were to: (i) identify whether there were any differences in the soil microbial communities, particularly those of the ammonia-oxidisers, beneath perennial ryegrass and Italian ryegrass, and (ii) determine whether such difference could explain the reduced N leaching loss observed in Lysimeter Experiment 1 (Chapter 3).

This experiment tested the following key hypothesis:

1. That Italian ryegrass decreases N leaching by inhibiting the first step of the nitrification process: ammonia oxidation.

4.2 Methodology

4.2.1 Experiment description and preparation

Soil was collected (0 to 0.15 m depth) for the experiment from the Lincoln University Research Dairy Farm, Canterbury, New Zealand (43°38'32.02" S, 172°27'44.94" E) on 21 November 2014 (Appendix A, Figure A 1). The soil type is described in detail in Section 3.2.1. Soil was well mixed with a spade and broken up by hand to remove large clods/structural units. On 12-13 December 2014 small pots (0.0144 m^2 surface area; 0.13 m deep) were filled with soil and placed ~0.1 m deep in a prepared sand bed with sand between the pots (Plate 4.1). This provided free drainage from the bottom and insulation similar to a field environment. The grass species were sown into appropriate pots at a rate of 20 kg ha^{-1} on 17-18 December 2014. An irrigation system with an oscillating sprinkler was set up using a Hozelock Water Timer. This applied water either once or twice daily during the cooler times of the day, and aimed to apply ~5-6 mm per day to meet demands from evapotranspiration.

4.2.2 Treatments and experimental design

Treatments are summarised in Table 4.1. A factorial design was implemented which consisted of forage type (3 levels: bare soil (no plants), perennial ryegrass (perennial RG), Italian ryegrass (Italian RG)) x urine (2 levels: with and without urine at 700 kg N ha^{-1}) x 5 sampling occasions (1, 15, 30, 61 and 90

days following treatment application) arranged in a randomised complete block design with four replicates. Each replicate block consisted of 30 pots arranged in a 3 by 10 rectangle. Blocks were arranged in a 2 by 2 block square (Plate 4.1).

Table 4.1 Pot experiment treatments.

Treatment no.	Forage type	Treatment	Replication	Cultivar
T1	Bare soil	Control	4	
T2	Bare soil	Urine	4	
T3	Perennial ryegrass (Perennial RG)	Control	4	Expo (AR1)
T4	Perennial ryegrass (Perennial RG)	Urine	4	Expo (AR1)
T5	Italian ryegrass (Italian RG)	Control	4	Tabu
T6	Italian ryegrass (Italian RG)	Urine	4	Tabu

Plate 4.1 Pot experiment layout in sand bed located at Lincoln University's Field Research Centre.

Treatment application

On 5 May 2015 (exactly one year after Lysimeter Experiment 1 (Chapter 3)), herbage in the pots was cut to a residual height of 50 mm. Fresh cow urine was collected during the afternoon milking from Friesian-Jersey-cross (KiwiCross^{TM}) cows that had been grazing perennial ryegrass-white clover at the Lincoln University Dairy Farm. A urine sample was collected, and was analysed overnight for N concentration on an Elementar Vario-Max CN Elemental Analyser (Elementar GmbH, Hanau, Germany). Urine was found to have a concentration of 3.94 g N L^{-1}. The next day, urea and glycine (9:1

ratio) were added to adjust the N concentration to 7 g N L^{-1}. The glycine was used to represent the amino acid fraction of urine and better mimic the actions of real urine (Fraser *et al.*, 1994). The urine was mixed thoroughly and 140 mL was applied to appropriate pots, this represented a rate of 700 kg N ha^{-1} which is typical of a cow urine patch (average 613 kg N ha^{-1}, range 200-2000 kg N ha^{-1} (Selbie *et al.*, 2015)). Control pots received 140 mL of water for consistency.

4.2.3 Pot maintenance

Soil testing and fertiliser applications

A soil test was conducted to determine nutrient status and pH of the soil prior to the experiment starting (Table 4.2). Based on these soil test results, pots received 1071 kg ha^{-1} of sulphur super 30 (0:7:0:30) as maintenance fertiliser on 2 March 2015 prior to treatment application. Nitrogen was applied as urea on 23 April 2015 to all pots at a rate of 25 kg N ha^{-1} to be consistent with Lysimeter Experiment 1 (Chapter 3). Due to the small surface area of the pots, this was applied in a liquid form using a syringe (10 mL of 7.73 g urea L^{-1} stock solution per pot).

Table 4.2 Soil test results of the collected soil.

	Pot experiment soil
pH	5.8
Olsen P (µg g^{-1})	16.8
Organic Matter (g kg^{-1})	33
Total C (g kg^{-1})	19.2
Total N (g kg^{-1})	1.6
Sulphate S (µg g^{-1})	3
CEC[1] ($cmol_c$ kg^{-1})	13
Exchangeable Ca^{2+} ($cmol_c$ kg^{-1})	6.8
Exchangeable Mg^{2+} ($cmol_c$ kg^{-1})	0.57
Exchangeable K^+ ($cmol_c$ kg^{-1})	0.28
Exchangeable Na^+ ($cmol_c$ kg^{-1})	0.21
BS[2] (%)	61.1

[1]Cation exchange capacity; [2]Base saturation

Forage management

Herbage was cut and discarded once it had reached the 3 leaf stage or ~3000 kg DM ha^{-1} to simulate grazing. This was consistent with Lysimeter Experiment 1 (Chapter 3).

4.2.4 Soil sampling

Selected pots were destructively harvested 1, 15, 30, 61, and 90 days post treatment application and soil samples were collected for subsequent soil chemistry and microbiology measurements. First the soil was removed from the pot and broken up, then any loose soil was shaken off and discarded (Plate 4.2). A sample of the rhizosphere soil (soil attached to the roots) was collected by vigorously shaking the soil from the roots and gently brushing soil off the roots. This soil was homogenised, and a subsample stored at -80°C prior to soil microbiology measurements. The remainder of the soil was stored at 4°C prior to soil chemistry measurements.

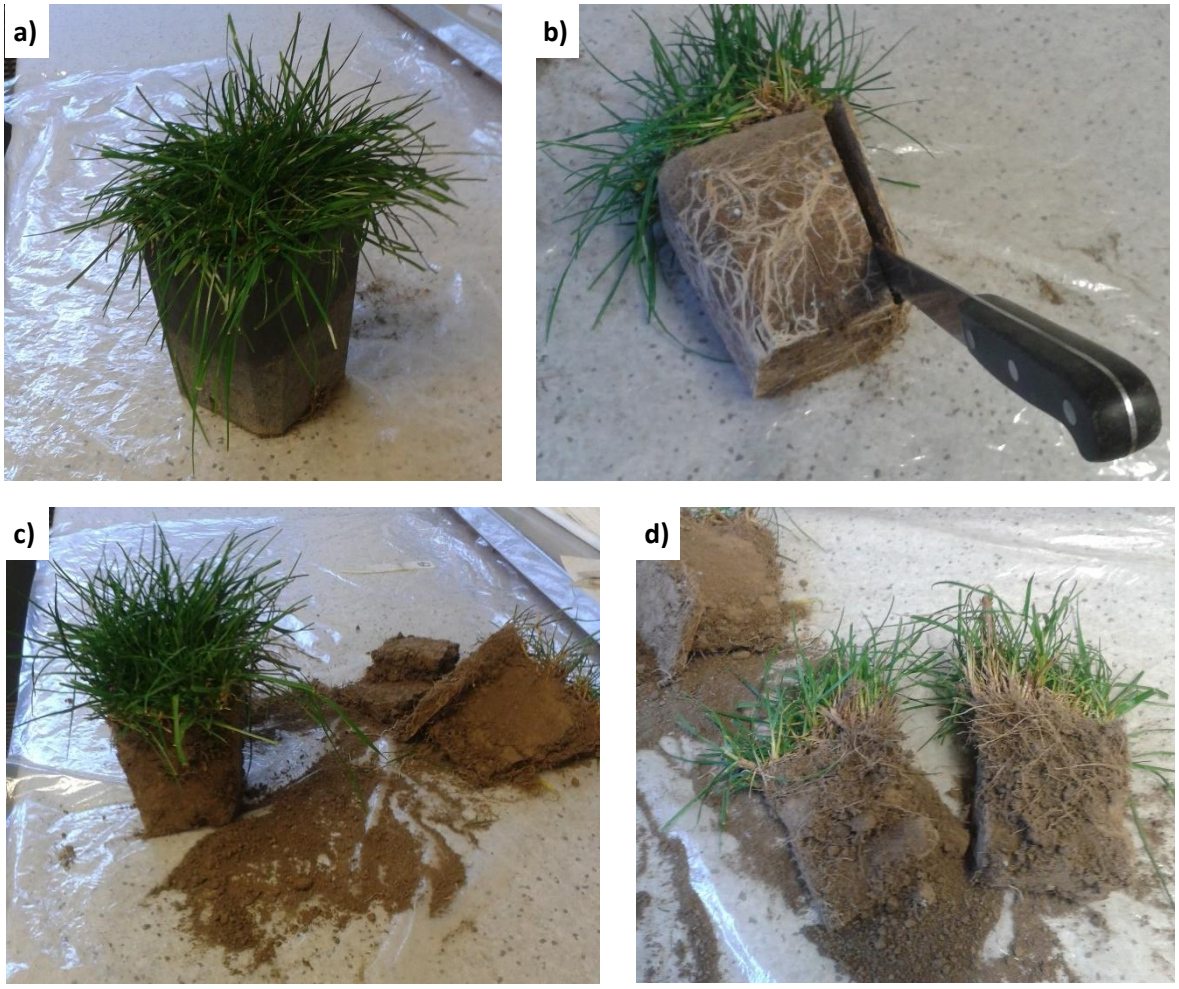


Plate 4.2 Soil sample collection: a) the pots from the field, b) and c) edges trimmed off, and d) soil broken apart then loose soil shaken off.

4.2.5 Soil microbiology measurements

DNA extraction and quality check of extracted DNA

DNA was extracted from a 0.25 g sample of fresh soil (where possible, otherwise from frozen soil) using a NucleoSpin® Soil Kit (Macherey-Nagel, Düren, Germany) as per the manufacturer's instructions, and is described in the section below. In the final step, DNA was eluted in 100 µL of Buffer SE (Macherey-

Nagel, Düren, Germany). This extracted DNA was frozen (-20°C) prior to further analysis. Agarose gel electrophoresis and NanoDrop analysis (Model: ND-1000, NanoDrop Technologies, DE, USA) were used to check the quantity and quality of the extracted DNA. Samples tested on the NanoDrop with a 260:280 of <1.4 were re-extracted. For the gel electrophoresis, a 1.5% agarose gel with RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology Inc.) was used with Tris/Borate/Ethylenediaminetetraacetic acid (TBE) buffer. A 5x DNA Loading Buffer (Bioline) was added to 5 µL of sample DNA. A DNA molecular marker (HyperladderI, Bioline) was included with each run to aid with size estimation. Gels were run for 45 minutes at 100 V (ENDURO™ Power Supplies 300V, Labnet International Inc., NJ, USA), and then photographed under UV light.

NucleoSpin® Soil Kit protocol for DNA extraction

For each extraction a 0.25 g soil sample was weighed into a NucleoSpin® Bead Tube, 700 µL of Buffer SL2, and 150 µL of Enhancer SX were added to the tube. This was processed using a FastPrep®-24 Sample Preparation System (M.P. Biomedicals, California, USA) at a speed of 6 m s^{-1} for two 30 s bursts with a 1-minute rest in between. The tubes were then centrifuged at 11000 x *g* for 2 minutes (Centrifuge 5424, Eppendorf AG, Hamburg, Germany). The supernatant was transferred into a sterilised 1.7 mL tube using a 1 mL pipette, 150 µL of Buffer SL3 was added. Samples were briefly vortexed or shaken for 5 s, incubated for 5 minutes at 4°C, then centrifuged at 11000 x *g* for 1 minute. Next, up to 700 µL of supernatant was transferred into a NucleoSpin® Inhibitor Removal Column fitted on top of a collection tube, and centrifuged at 11000 x *g* for 1 minute. This process was repeated with the remainder of the supernatant (when >700 µL), and then 250 µL of Buffer SB was added to the flow through and mixed with a pipette. A 550 µL sample of this was loaded onto a NucleoSpin® Soil Column on top of a clean collection tube, and centrifuged at 11000 x *g* for 1 minute. The flow through was discarded and this step was repeated with the remaining sample. Next 500 µL of Buffer SB was added to the NucleoSpin® Soil Column, centrifuged at 11000 x *g* and flow through was again discarded. This process was repeated with 550 µL of Buffer SW1, and then twice with 700 µL of Buffer SW2. After the final flow through was discarded, tubes were centrifuged at 11000 x *g* for a further 2 minutes to remove any residual ethanol. The NucleoSpin® Soil Column was transferred to a clean collection tube and 100 µL of Elution Buffer SE was added to the column. The DNA was eluted by incubating the sampes for 1 minute at room temperature and then centrifuging at 11000 x *g* for 30 s. This eluted DNA was then stored at -20°C for further analysis.

Real-time qPCR

Real-time quantitative polymerase chain reaction (qPCR) was used to determine the abundance of the *amoA* gene for AOB and AOA, as well as bacterial 16S and archaeal 16S rRNA genes in the total genomic DNA extracted from soil using the primer pairs (amoA-1F, amoA R-i; Arch-amoAF, Arch-amoAR; 1369F, 1492R; A364aF, A934bR) described in Table 4.3.

All qPCR reactions were prepared using a CAS1200 Robotic liquid handling system (Corbett Robotics, Australia) (Plate 4.3a). Real-time qPCR analysis was carried out using a Rotor-Gene™ 6000 (Corbett Research, Australia) (Plate 4.3b). Each well in the RotorDisc™ 100 contained 14.5 µL of master mix (containing 8 µL 2x SYBR® Premix Ex Taq™ (Tli RNaseH Plus, Takara Bio Inc., Shiga, Japan), 0.4 µL of each primer (this was 0.64 µL for AOB), and sterile deionised water to bring up to total volume of 14.5 µL), and 1.5 µL of DNA sample. Prior to qPCR all DNA were diluted 1 in 10 to minimise potential PCR inhibition which had previously been observed with undiluted DNA. Sterilised deionised water was used as a blank. Serial dilutions of standards with a range of 10^1 to 10^7 copies μL^{-1} were run in duplicate for each gene to produce standard curves. Several samples from different runs were included in following runs to ensure run-to-run consistency. Once the PCR reactions were prepared the RotorDisc™ 100 was sealed using a Gene-Disc™ Heat Sealer (HS-01, Corbett Research, Australia) (Plate 4.3c). Standard curves for real-time qPCR were developed using the following process: Bacterial and archaeal *amoA* and 16S genes were amplified from the extracted DNA using the aforementioned primers. A PCR clean up kit (Axygen) was then used to purify the PCR products which were then cloned into the pGEM-T Easy Vector (Promega, Madison, WI). Following the manufacturer's instructions, the resulting clones were transformed in *Escherichia coli* JM109 competent cells (Promega). The transformed *E. coli* cells were grown on solid LB plates at 37°C overnight. Ten to fifteen bacterial colonies from the plate were then individually inoculated into a 3 mL LB broth medium and incubated overnight in an orbital incubator- shaker at 37°C and 250 rpm. The plasmids carrying correct gene inserts were then extracted from bacterial cultures using QIA Prep Spin Miniprep Kit (Qiagen, Crawley, UK) and sent for sequencing. The plasmid DNA was used as template in a PCR reaction with *E. coli* T7 and SP6 primers and the PCR products purified as described earlier. The DNA concentration was determined on a Qubit™ Fluorometer (Invitrogen™, New Zealand). The copy numbers of target genes were then calculated directly from the concentration of purified DNA. To generate an external standard curve, tenfold serial dilutions of a known copy number of the PCR amplicons were then subjected to a real-time PCR assay in duplicate. The qPCR cycling conditions are described in Table 4.3. Each run was followed by a melt curve analysis which involved gradually increasing the temperature from 72°C to 99°C and monitoring the decrease in fluorescence intensity to check for nonspecific amplification products.

Table 4.3 Primers, standards and cycling conditions for PCR reactions.

	Gene	AOB[1] amoA		AOA[2] amoA		Bacteria 16S rRNA		Archaea 16S	
	Primer pairs (10 µM)	amoA-1F 5'-GGGGHTTYTACTGGTGGT-3' (Stephen *et al.*, 1999) amoA R-i 5'-CCCCTCNGNAAANCCTTCTTC-3' (Hornek *et al.*, 2006)		Arch-amoAF 5'-STAATGGTCTGGCTTAGACG-3' (Francis *et al.*, 2005) Arch-amoAR 5'-GCGGCCATCCATCTGTATGT-3' (Francis *et al.*, 2005)		1369F 5'-CGGTGAATACGTTCYCGG-3' (Suzuki *et al.*, 2000) 1492R 5'-GGWTACCTTGTTACGACTT-3' (Suzuki *et al.*, 2000)		A364aF 5'-CGGGGYGCASCAGGCGCGAA-3' (Burggraf *et al.*, 1997) A934bR 5'- GTGCTCCCCCGCCAATTCCT-3' (Großkopf *et al.*, 1998)	
	Standard	10^1 to 10^7		10^1 to 10^7		10^1 to 10^7		10^1 to 10^7	
	PCR efficiency	95-99%		94-100%		98-105%		96-97%	
# of cycles	Cycling conditions	Temp. (°C)	Time (s)	Temp. (°C)	Time (s)	Temp. (°C)	Time (s)	Temp. (°C)	Time (s)
1	Initial denaturation	94	120	94	120	94	120	94	120
40	Denaturation	94	20	94	20	94	10	94	20
	Primer annealing	57	30	55	30	56	30	58	30
	Extension*	72	30	72	30			72	20
	Final extension	72	180	72		56		72	

[1]Ammonia-oxidising bacteria, [2]Ammonia-oxidising archaea; *changes in fluorescence intensity were measured after each extension cycle at 85°C (for AOB and AOA), 56°C for bacterial 16S rRNA and 72°C for archaeal 16S rRNA genes.

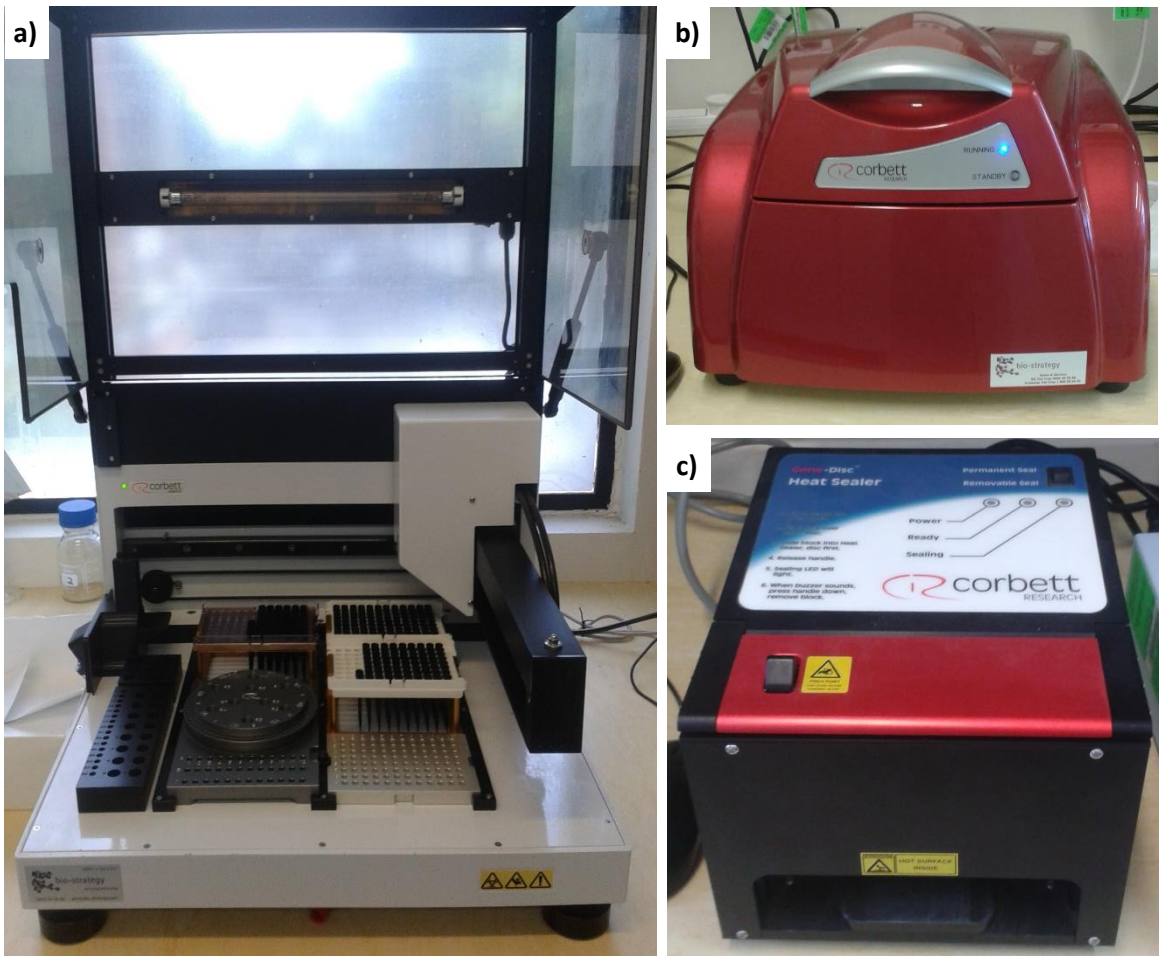


Plate 4.3 Equipment for real-time qPCR: a) the CAS1200 Robotic liquid handling system, b) the Rotor-Gene™ 6000, and c) the Gene-Disc™ Heat Sealer.

RNA extraction

RNA was extracted using the hexadecyltrimethylammonium bromide (CTAB) extraction protocols described by Griffiths *et al.* (2000). Firstly, a 10% solution of CTAB in 0.7 M sodium chloride (NaCl) was combined with an equal volume of 240 mM potassium phosphate buffer to form a 'modified CTAB buffer'. Soil samples (0.3 g) were weighed into NucleoSpin® Bead Tubes (Macherey-Nagel, Düren, Germany) and 0.5 mL of modified CTAB buffer added to each, followed by 0.5 mL of phenol:chloroform:isoamylalcohol (25:24:1). Tubes were shaken using the MP FastPrep®-24 Sample Preparation System (MP Biomedicals, USA) at a speed of 6 m s^{-1} for three lots of 40 seconds. In between each run, samples were put onto ice for 2 minutes to cool. Samples were then centrifuged at 16 000 x *g* for 5 minutes at 4°C (Prism™ R Refrigerated Microcentrifuge, Labnet International Inc., NJ, USA). The top aqueous layer was pipetted into a clean tube and an equal volume of chloroform:isoamylalcohol (24:1) was added. Tubes were mixed well, then centrifuged at 16 000 x *g* for 5 minutes at room temperature (Centrifuge 5424, Eppendorf AG, Hamburg, Germany). The top aqueous layer was again removed into a clean 1.5 mL tube and two volumes of 30% polyethylene glycol (PEG) in 1.6 M NaCl

solution was added. Tubes were well mixed then incubated at 4°C for 3 hours. Next samples were centrifuged at 18 000 x *g* for 10 minutes at 4°C to form a small insoluble pellet. The solution was carefully removed, and the pellet then washed with ice-cold 70% ethanol and centrifuged (18 000 x *g*, 4°C) for a further 10 minutes. The ethanol was carefully discarded and the pellet dried at room temperature for 5 minutes. The pellet was resuspended in 25 µL of diethyl pyrocarbonate treated water. Samples were then stored at -80°C. Gel electrophoresis was carried out, using the same method as described following DNA extraction (except the gel was run at 80 V for 35 minutes), to check the success of the extraction prior to DNase treatment.

DNase treatment and inhibitor removal

The extracted RNA samples were treated with DNase to remove any DNA using a TURBO DNA-*free*™ Kit (ThermoFisher Scientific, MA, USA) following manufacturer's instructions. First 10 µL of each extracted RNA sample was combined with 7 µL of DEPC-treated water, 2 µL of 10x TURBO DNase Buffer, and 1 µL of TURBO DNase, gently mixed together with a pipette, then incubated at 37°C for 30 minutes. Next, 2 µL of DNase Inactivation Reagent was added to the tube and incubated for 2 minutes at room temperature, mixed occasionally. Tubes were centrifuged at 10000 x *g* for 1.5 minutes (Centrifuge 5424, Eppendorf AG, Hamburg, Germany) and the top aqueous layer was transferred into a fresh 200 µL tube. Zymo-Spin™ IV-HRC columns (Zymo Research Corporation, California, USA), were prepared by snapping off the base, these were inserted into a collection tube and centrifuged at 8000 x *g* for 3 minutes. The RNA sample was transferred into the Zymo-Spin™ IV-HRC column and centrifuged at 8000 x *g* for 1 minute. This DNase treated RNA was then tested for DNA contamination using real-time qPCR analysis (Rotor-Gene™ 6000, Corbett Research, Australia). These were set up using a CAS1200 Robotic liquid handling system (Corbett Robotics, Australia) as described earlier for the AOB *amoA* gene. For samples which showed the presence of DNA, a second DNase treatment was necessary. This involved combining ~6 µL of RNA sample with 2 µL of 10x TURBO DNA-free Second Digest Buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 5 mM $CaCl_2$, nuclease-free water), 1 µL of DNase, and 11 µL of water. These were gently mixed and incubated at 37°C for 1 hour. Next, 2 µL of DNase Inactivation Reagent was added to the tube and incubated for 2 minutes at room temperature, mixed occasionally. Tubes were centrifuged at 10000 x *g* for 1.5 minutes and the top aqueous layer was transferred into a fresh 200 µL tube. Samples were again tested for DNA contamination using real-time qPCR analysis (as previously described).

Once all the samples were free of DNA contamination they were analysed for RNA levels using a Qubit® RNA HS kit and a Qubit® 1.0 Fluorometer (Invitrogen™, New Zealand). First a master mix was made up at 199:1 (buffer:dye) ratio. A 2 µL RNA sample was added to 198 µL of this master mix dye solution in a 0.5 mL tube, vortexed briefly and incubated at room temperature for 2 minutes. Tubes were then placed into the Qubit™ Fluorometer and concentration of RNA measured (ng μL^{-1}) (Plate 4.4).

Plate 4.4 The Qubit™ Fluorometer used to determine RNA concentration.

cDNA synthesis

Once the presence and quality of RNA was confirmed by Qubit™ Fluorometer (Invitrogen™, New Zealand), this RNA was used for complementary DNA (cDNA) synthesis. First 1 µL of random primers (50-250 ng), 1 µL of 10 mM dNTP Mix, 13 µL of nuclease-free ddH_2O, and 5 µL of RNA sample were added to a nuclease-free microcentrifuge tube. These were heated for 5 minutes at 65°C and cooled on ice for at least 2 minutes. Tubes were centrifuged briefly to collect contents at the bottom of the tube. Then 4 µL of 5X First Strand Buffer, 1 µL of 0.1 M DTT, 1 µL of RNaseOUT™ Recombinant RNase Inhibitor (40 units μL^{-1}), and 1 µL of SuperScript™ III RT (200 units μL^{-1}) were added to the tube and mixed gently with a pipette. Tubes were then incubated for 5 minutes at 25°C, then 50 minutes at 50°C, and inactivated by incubating for 15 minutes at 70°C. Real-time qPCR analysis was carried out to quantify bacterial and archaeal *amoA* genes using the same methodology as previously described.

4.2.6 Soil chemistry measurements

On each of the sampling dates, soil was also tested for soil chemistry parameters such as soil moisture content, pH, and ammonium and nitrate concentration.

Moisture

Soil moisture content was determined by weighing 10-20 g of soil into a metal dish. The sample was then oven dried for 24 hours at 105°C, and re-weighed to determine the dry weight. Moisture content was determined as follows:

Moisture content (%) = (Moist soil (g) - Dry soil (g))/Dry soil (g) x 100

Soil pH

Soil pH was determined by weighing a 15 g sample of field-moist soil into a 70 mL vial with 25 mL of deionised water. This was stirred well and left overnight to stabilise. Soil pH was read using a calibrated pH meter (SevenEasy pH, Mettler-Toledo AG, Switzerland).

Soil ammonium and nitrate concentration

The soil NH_4^+-N and NO_3^--N concentrations were determined by weighing out 5 g of field-moist soil into a 50 mL plastic centrifuge tube containing 25 mL of 2 M KCl. This was shaken for 1 hour (Ratek Platform Mixer, Model: RM2, Victoria, Australia), centrifuged at 4000 rpm for 10 minutes (Heraeus Multifuge 3S-R Centrifuge, Thermo Electron Corporation, Germany) and then filtered through Advantec 5C 110 mm filter paper (adapted from Blakemore *et al.*, 1987). Samples were stored in a freezer (-20°C) prior to being analysed for NO_3^--N and NH_4^+-N concentrations by flow injection analysis using a FOSS FIAstar 5000 twin channel analyser (Foss Tecator AB, Hoganas, Sweden). This is described in more detail in Section 3.2.6.

4.2.7 Statistical analysis

Data were analysed using Genstat (18th Edition, VSN International Ltd.) by conducting an analysis of variance (ANOVA) as a 3 (forage type) x 2 (treatment) x 5 (sampling occasions) factorial with four blocks (randomised block design). For the sampling occasions factor, polynomial contrasts were included in the ANOVA. While for the forage type factor, two orthogonal contrasts were specified: a) comparing the plants with bare soil and b) comparing perennial RG with Italian RG. The variables AOB, AOA, Bac16S, Archea16S, soil NH_4^+-N, and soil NO_3^--N were log-transformed to achieve homogeneity of variance. Soil moisture and soil pH were not transformed. Weighted averages (AOB, AOA, Bac16S, and Archaea16S) and RNA data (AOB, and AOA) for day 61 were log-transformed and analysed in a similar manner, there was no sampling occasion factor for these. Where significant effects were shown, the unrestricted LSD procedure (Saville, 1990) at the 5% level was used to identify differences among means. Where log transformations were used for statistical analysis, log means are displayed in tables with LSDs, and for graphs, data were back-transformed using anti-log to be more easily compared with other studies.

4.3 Results

4.3.1 Climate conditions and water inputs

During the experimental period (6 May 2015 to 4 August 2015), the mean daily air temperature ranged from a low of -0.4°C in July 2015 to a high of 20.9°C at the beginning of the experimental period in May 2015 (Figure 4.1a). Similarly, daily mean soil temperature (10 cm depth) ranged from 1.8°C (July 2015) to 15.7°C (May 2015) (Figure 4.1a). Total water inputs for the 3-month experimental period were 142

mm, all of which was rainfall since irrigation was not applied during the experimental period (Figure 4.1b).

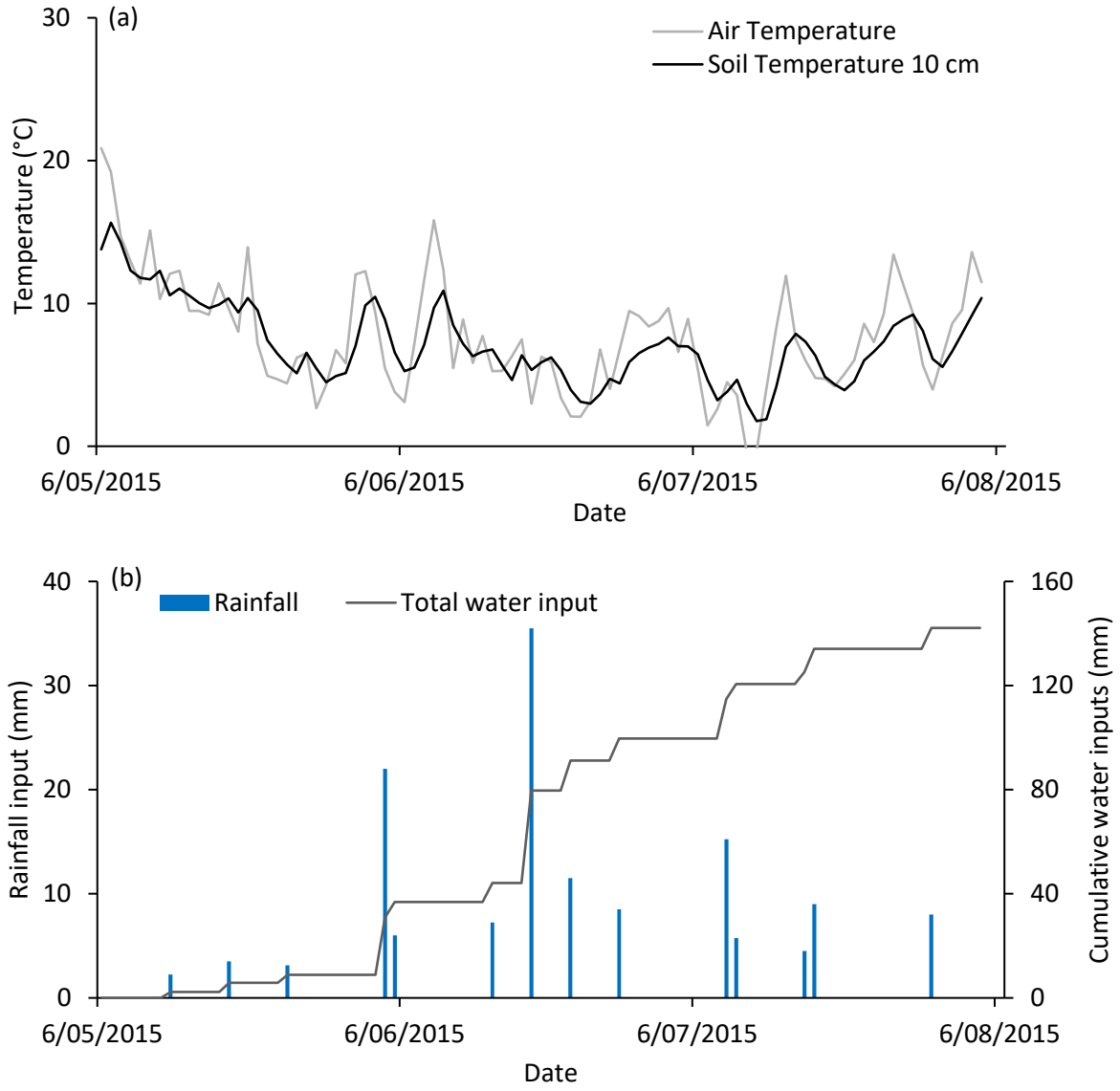


Figure 4.1 a) Average daily air temperature and soil temperature (at 10 cm), and b) daily and cumulative rainfall inputs for the experimental period: 6 May 2015 to 4 August 2015.

4.3.2 Soil microbiology

Ammonia-oxidising bacteria (AOB)

Copy numbers of the AOB *amoA* gene ranged from 8.53 x10^6 to 7.21 x10^7 throughout the experiment (Figure 4.2). The application of urine increased the abundance of the AOB *amoA* gene (Figure 4.3), this was particularly evident in the bare soil pots from day 15 onwards (Figure 4.2). Both ryegrass treatments reduced the abundance of AOB *amoA* in urine-treated soil, compared with bare soil ($P < 0.05$) (Figure 4.2, Figure 4.3). There were no differences in the abundance of the AOB *amoA* when ryegrass treatments were compared (Figure 4.2, Figure 4.3). There was no interaction between urine treatment and forage type. Log-transformed means and LSDs are shown in Table 4.4 and Table 4.5.

The transcription activity of the AOB *amoA* gene in the soil 61 days after treatment application, as measured by the RNA copy numbers, increased by 9, 4, and 15 times with the application of urine, compared with the Control for bare soil, perennial RG, and Italian RG, respectively (Figure 4.4). The expression of the AOB *amoA* gene was by far the highest in the urine-treated bare soil with 1.3 $x10^6$ copies μg^{-1} RNA, compared with 5.0 $x10^5$ and 4.1 $x10^5$ copies μg^{-1} RNA, for urine-treated perennial RG and Italian RG, respectively (Figure 4.4). Log-transformed means and LSDs are shown in Table 4.6.

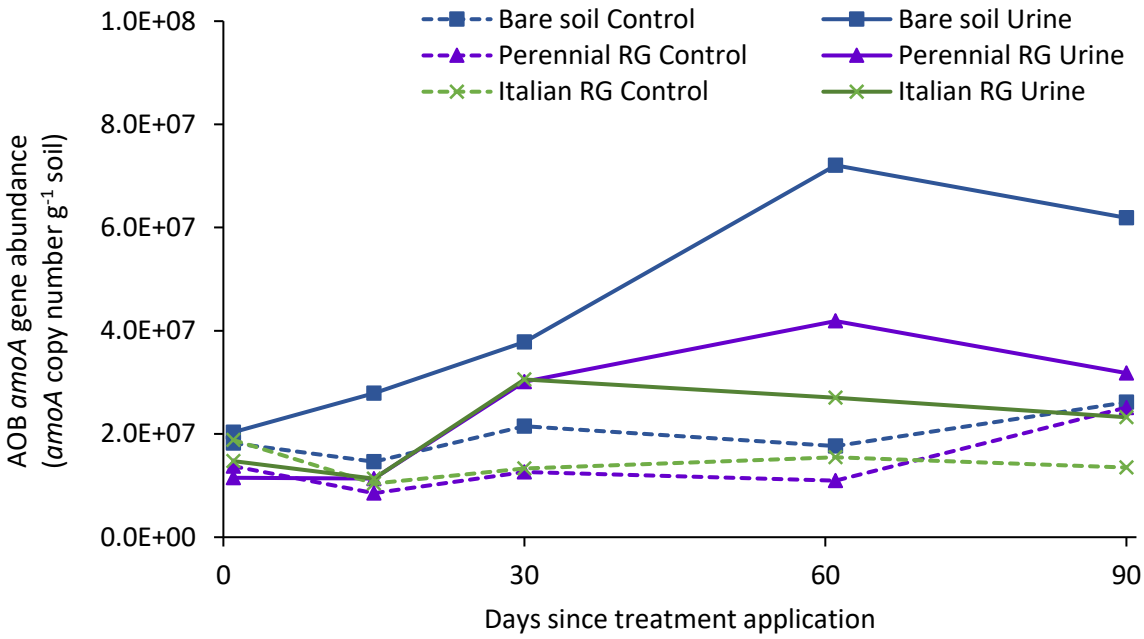


Figure 4.2 Back-transformed ammonia-oxidising bacteria (AOB) *amoA* gene abundance after 1, 15, 30, 61, and 90 days following treatment application with or without urine (700 kg N ha^{-1}) in May 2015.

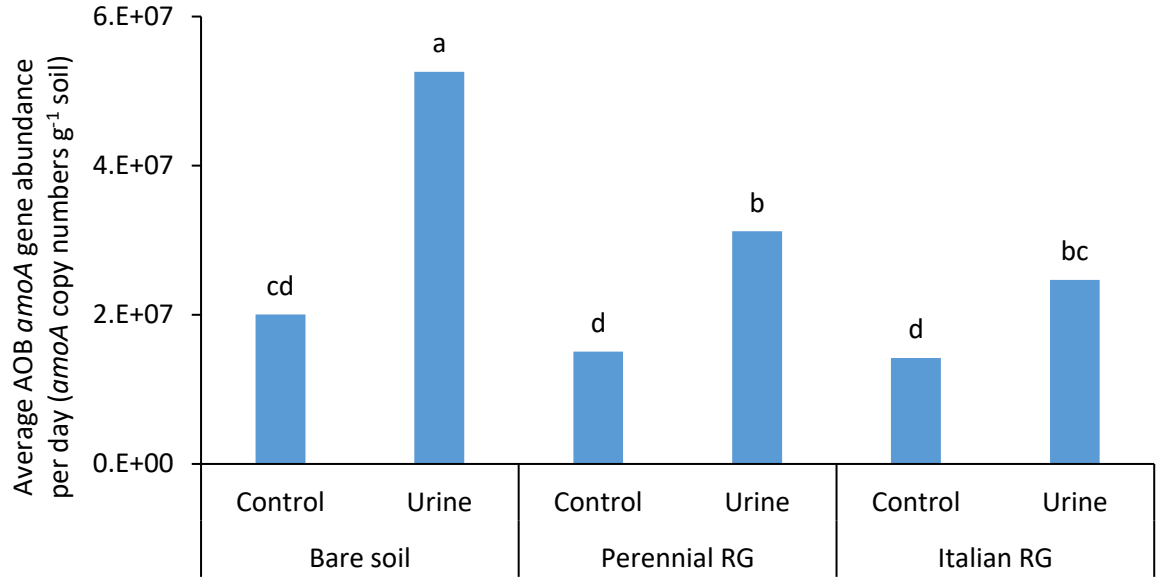


Figure 4.3 Back-transformed weighted average for ammonia-oxidising bacteria (AOB) *amoA* gene abundance per day. Treatments (Control, and Urine at 700 kg N ha^{-1}) were applied in May 2015. Bars with the same letter (a-d) are not significantly different at the 5% level.

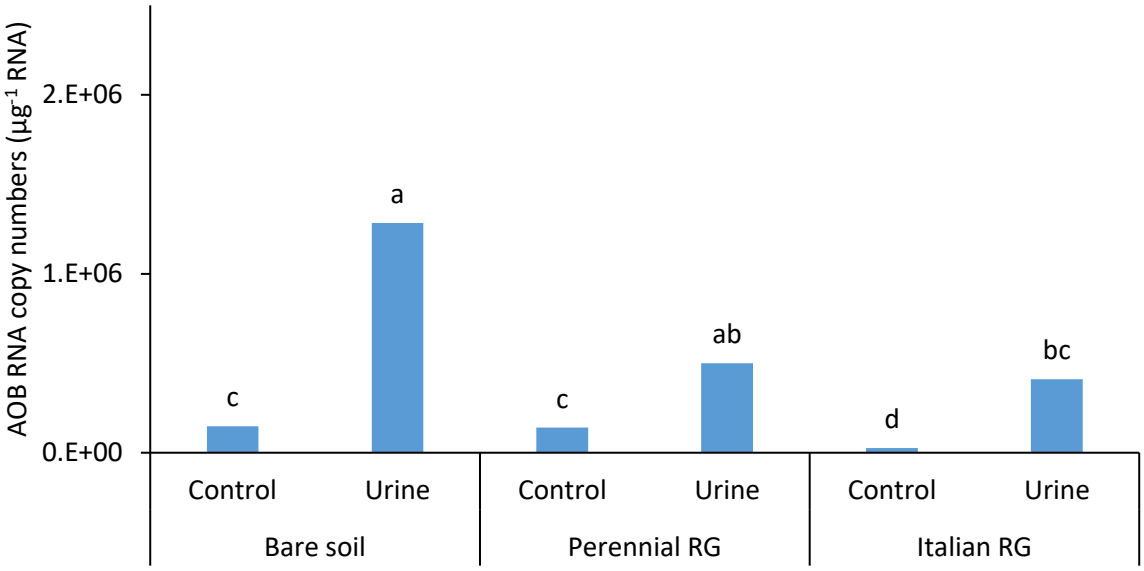


Figure 4.4 Back-transformed RNA copy numbers of ammonia-oxidising bacteria (AOB) in the soil after 61 days. Treatments (Control, and Urine at 700 kg N ha^{-1}) were applied in May 2015. Bars with the same letter (a-d) are not significantly different at the 5% level.

Ammonia-oxidising archaea (AOA)

Copy numbers of the AOA *amoA* gene (2.44 x10^{6}-1.29 x10^{7}) were much lower than that of the AOB *amoA* gene. In contrast to AOB, the abundance of the AOA *amoA* gene did not increase with the application of urine (Figure 4.5, Figure 4.6). Bare soil had a higher abundance of AOA *amoA* than perennial RG or Italian RG ($P < 0.05$) (Figure 4.6). There were no differences between the abundance of the AOA *amoA* in the perennial RG pots and the Italian RG pots (Figure 4.5, Figure 4.6). Log-transformed means and LSDs are shown in Table 4.4 and Table 4.5.

In contrast to AOB, the transcription activity of the AOA *amoA* gene, as measured by the RNA copy numbers in the soil, was not increased by the application of urine after 61 days (Figure 4.7). There were no significant differences in the AOA RNA copy numbers of the perennial RG and Italian RG treatments with or without urine application (Figure 4.7). Log-transformed means and LSDs are shown in Table 4.6.

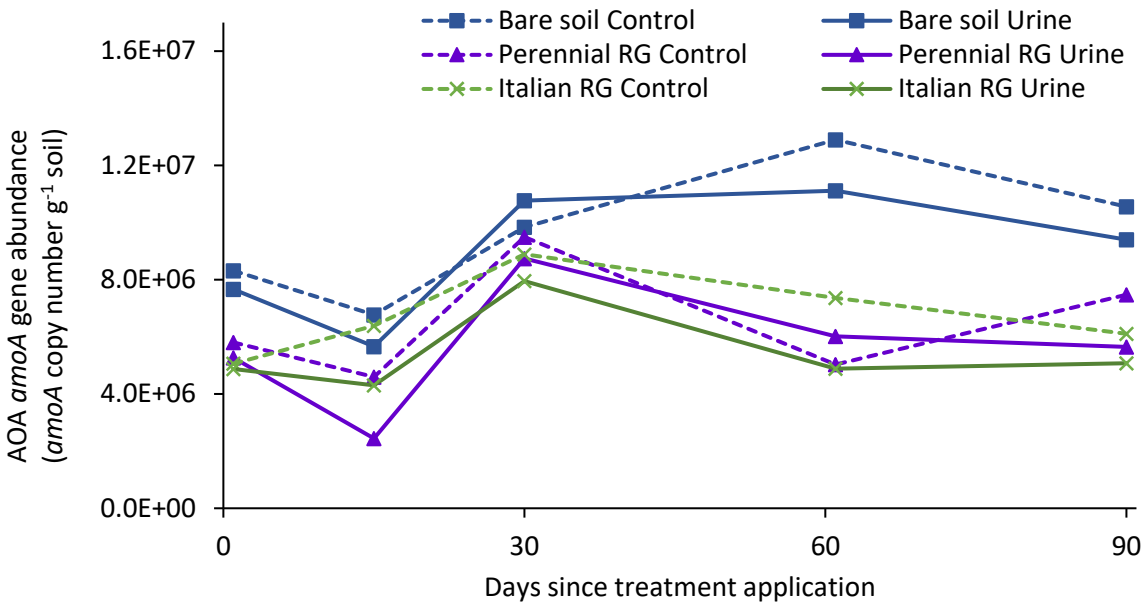


Figure 4.5 Back-transformed ammonia-oxidising archaea (AOA) *amoA* gene abundance after 1, 15, 30, 61, and 90 days following treatment application with or without urine (700 kg N ha^{-1}) in May 2015.

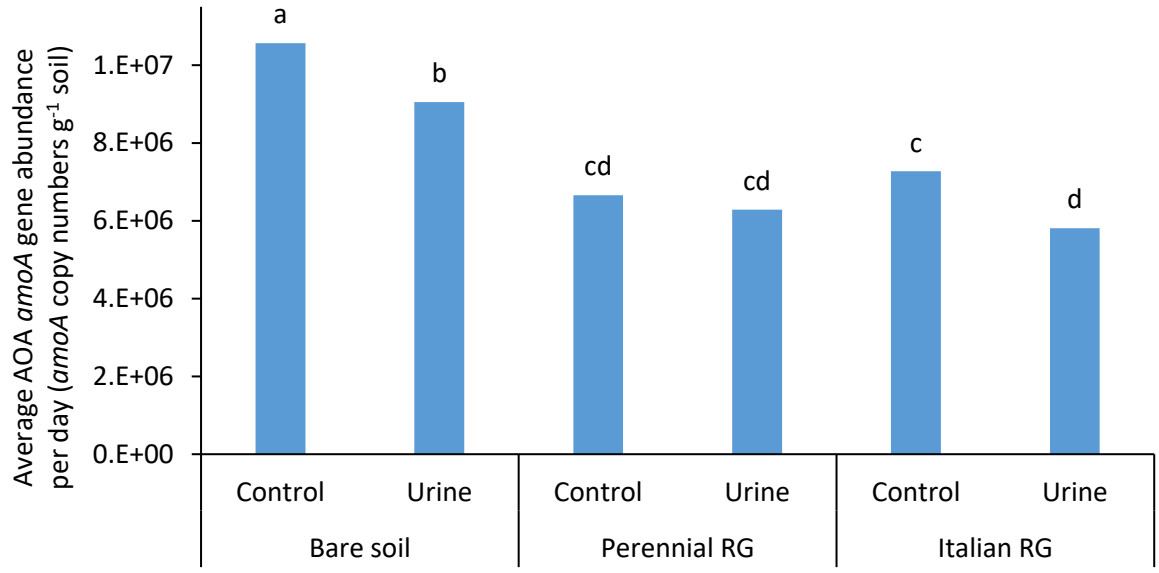


Figure 4.6 Back-transformed weighted average for ammonia-oxidising archaea (AOA) *amoA* gene abundance per day. Treatments (Control, and Urine at 700 kg N ha^{-1}) were applied in May 2015. Bars with the same letter (a-d) are not significantly different at the 5% level.

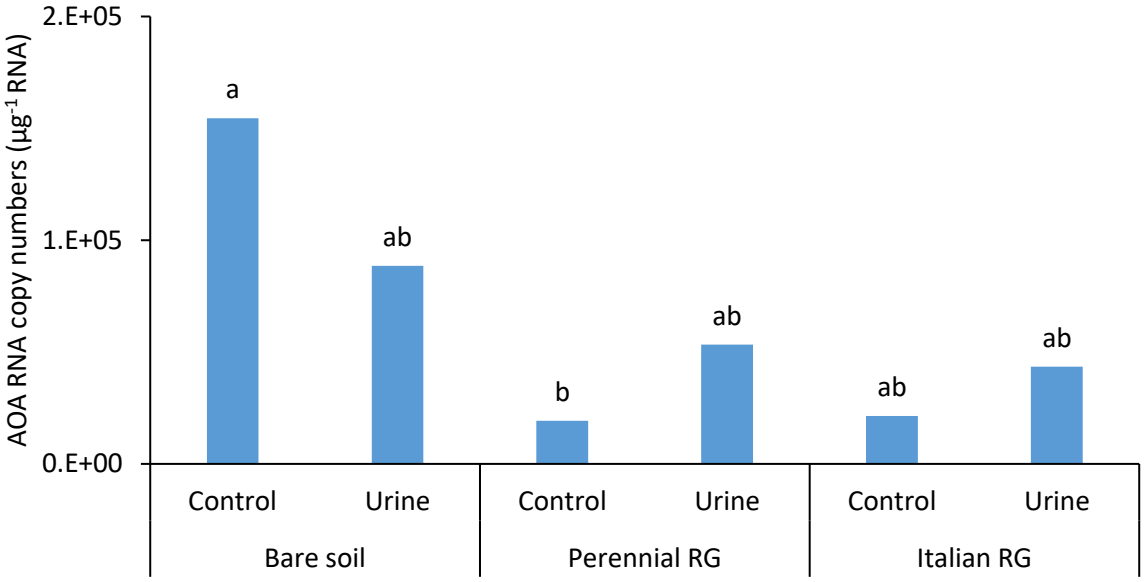


Figure 4.7 Back-transformed RNA copy numbers of ammonia-oxidising archaea (AOA) in the soil after 61 days. Treatments (Control, and Urine at 700 kg N ha^{-1}) were applied in May 2015. Bars with the same letter (a-b) are not significantly different at the 5% level.

The abundance of the bacteria 16S gene ranged from 1.73 $x10^9$ to 5.14 $x10^9$ and was not affected by forage type or urine treatment throughout the experimental period (Figure 4.8, Figure 4.9). However, time had an effect, and there was a significant interaction between urine treatment and time (P = 0.024). Average bacteria 16S gene abundance for urine-treated pots was significantly lower for both ryegrasses, compared with bare soil (Figure 4.9). Log-transformed means and LSDs are shown in Table 4.4 and Table 4.5.

The gene copy numbers of the archaea 16S gene were much lower than the bacteria 16S genes, and ranged from 3.39 $x10^7$ to 1.45 $x10^8$ (Figure 4.10). Archaea 16S gene abundance was affected by forage type but not urine treatment throughout the experimental period. For bare soil, the abundance of the archaea 16S gene was higher than in both the ryegrass treatments (P < 0.05) (Figure 4.10, Figure 4.11). Log-transformed means and LSDs are shown in Table 4.4 and Table 4.5.

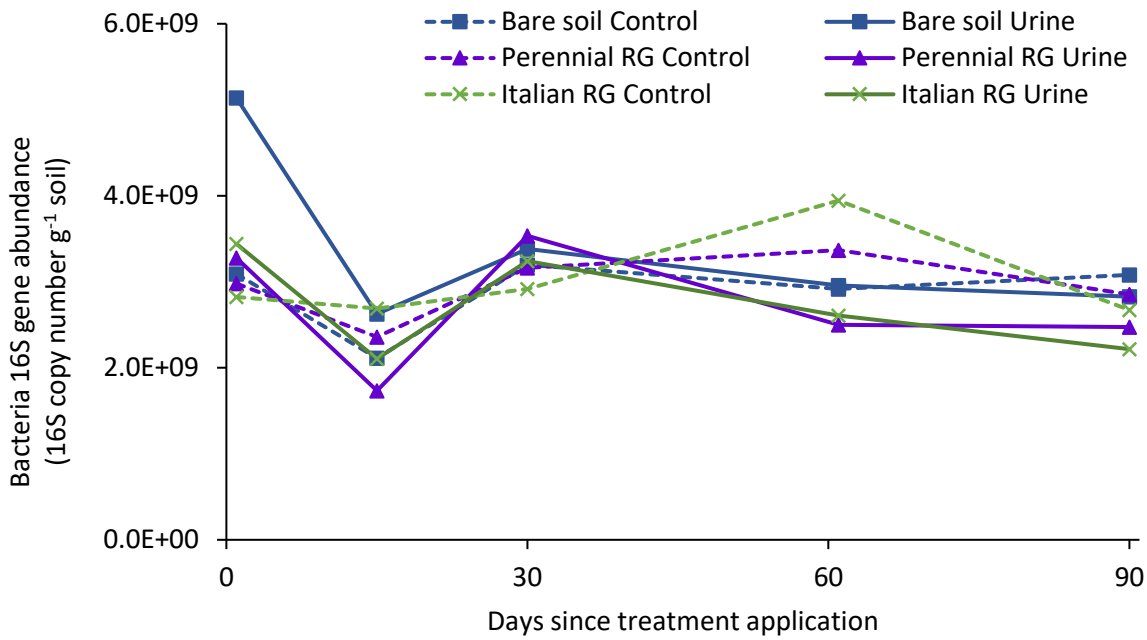


Figure 4.8 Back-transformed bacteria 16S gene abundance after 1, 15, 30, 61, and 90 days following treatment application with or without urine (700 kg N ha^{-1}) in May 2015.

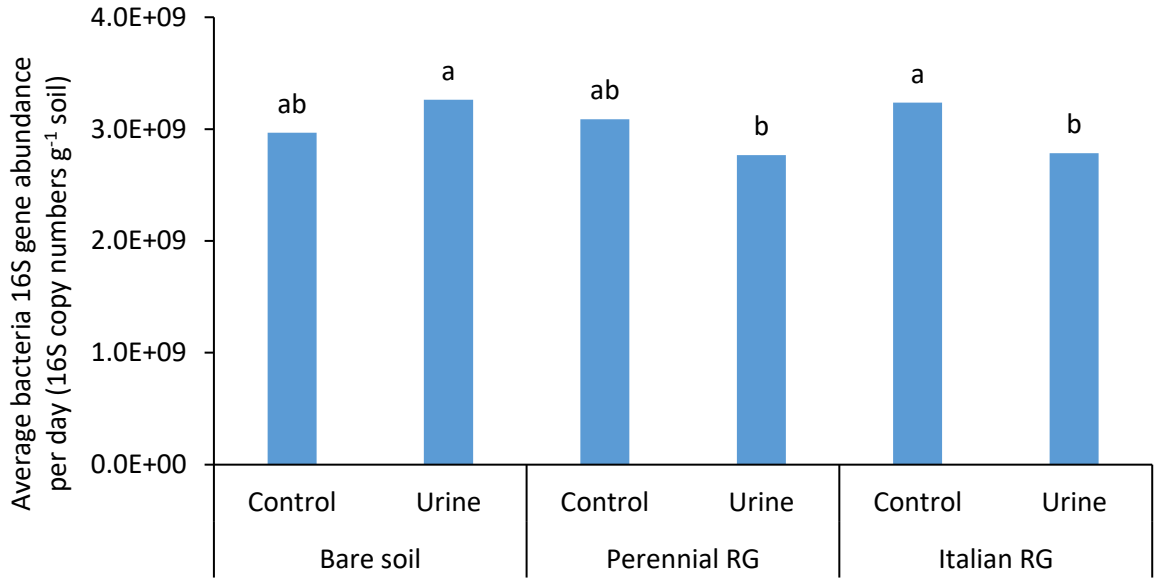


Figure 4.9 Back-transformed weighted average for bacteria 16S gene abundance per day. Treatments (Control, and Urine at 700 kg N ha^{-1}) were applied in May 2015. Bars with the same letter (a-b) are not significantly different at the 5% level.

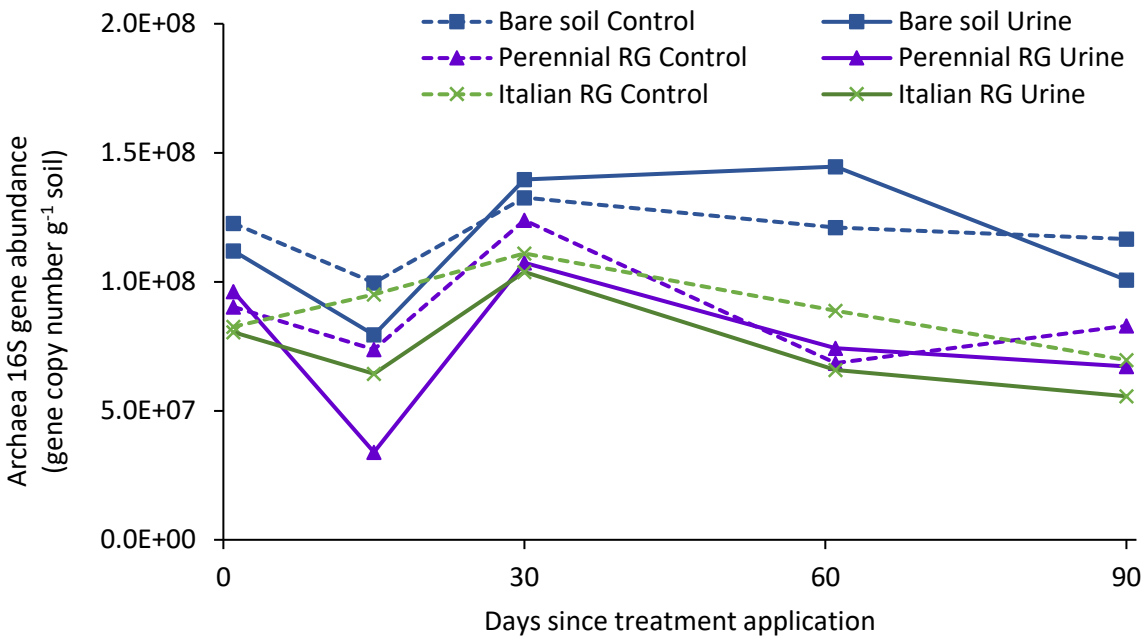


Figure 4.10 Back-transformed archaea 16S gene abundance after 1, 15, 30, 61, and 90 days following treatment application with or without urine (700 kg N ha^{-1}) in May 2015.

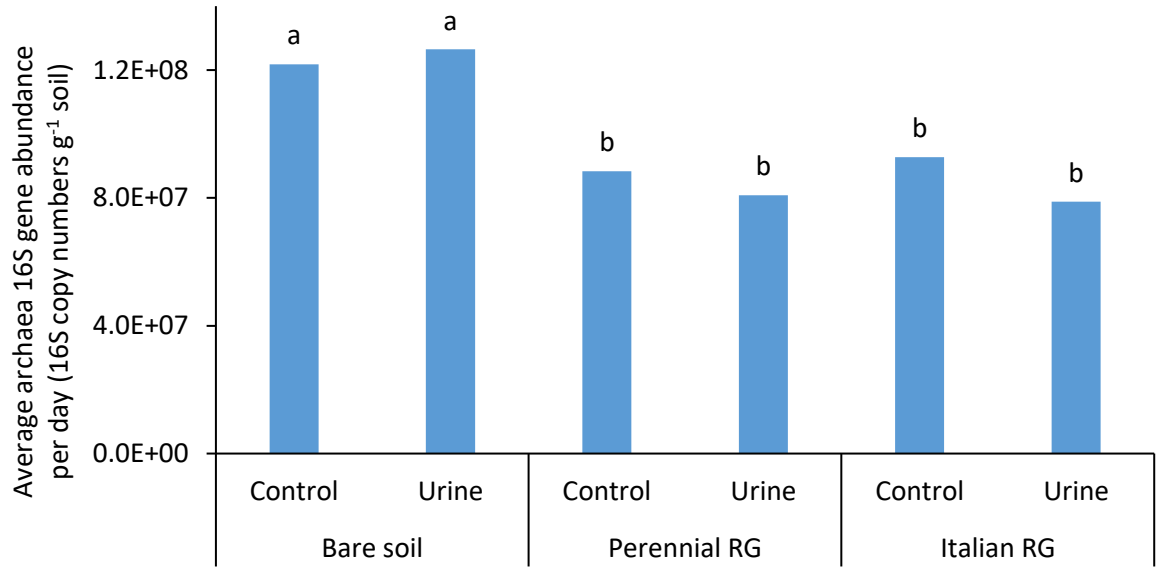


Figure 4.11 Back-transformed weighted average for archaea 16S gene abundance per day. Treatments (Control, and Urine at 700 kg N ha^{-1}) were applied in May 2015. Bars with the same letter (a-b) are not significantly different at the 5% level.

Table 4.4 Log-transformed means and LSDs for ammonia-oxidising bacteria (AOB) and ammonia-oxidising archaea (AOA) *amoA*, and bacteria and archaea 16S gene abundance following treatment application with or without urine (700 kg N ha^{-1}) in May 2015.

	Forage type	Treatment	Day 1	Day 15	Day 30	Day 61	Day 90
AOB *amoA*	Bare soil	Control	7.260	7.165	7.332	7.247	7.417
	Bare soil	Urine	7.309	7.445	7.578	7.858	7.792
	Perennial RG	Control	7.138	6.931	7.102	7.040	7.400
	Perennial RG	Urine	7.061	7.055	7.479	7.622	7.502
	Italian RG	Control	7.275	7.017	7.125	7.190	7.131
	Italian RG	Urine	7.168	7.055	7.485	7.431	7.366
	LSD (5%)	*0.2815*					
AOA *amoA*	Bare soil	Control	6.920	6.831	6.993	7.111	7.024
	Bare soil	Urine	6.884	6.753	7.032	7.046	6.973
	Perennial RG	Control	6.764	6.662	6.977	6.701	6.874
	Perennial RG	Urine	6.722	6.388	6.942	6.779	6.752
	Italian RG	Control	6.704	6.806	6.949	6.867	6.786
	Italian RG	Urine	6.688	6.634	6.900	6.689	6.706
	LSD (5%)	*0.1928*					
Bacteria 16S	Bare soil	Control	9.490	9.325	9.505	9.465	9.488
	Bare soil	Urine	9.711	9.419	9.529	9.471	9.451
	Perennial RG	Control	9.474	9.373	9.500	9.527	9.456
	Perennial RG	Urine	9.515	9.239	9.548	9.398	9.393
	Italian RG	Control	9.451	9.430	9.465	9.596	9.427
	Italian RG	Urine	9.537	9.323	9.510	9.416	9.345
	LSD (5%)	*0.1745*					
Archaea 16S	Bare soil	Control	8.089	7.998	8.123	8.083	8.067
	Bare soil	Urine	8.049	7.900	8.145	8.160	8.003
	Perennial RG	Control	7.956	7.868	8.093	7.836	7.919
	Perennial RG	Urine	7.983	7.531	8.031	7.871	7.828
	Italian RG	Control	7.917	7.978	8.045	7.949	7.843
	Italian RG	Urine	7.906	7.809	8.016	7.819	7.745
	LSD (5%)	*0.2234*					

Table 4.5 Log-transformed weighted averages and LSDs for ammonia-oxidising bacteria (AOB) and ammonia-oxidising archaea (AOA) *amoA*, and bacteria and archaea 16S gene abundance per day following treatment application with or without urine (700 kg N ha^{-1}) in May 2015.

		AOB	AOA	Bacteria 16S	Archaea 16S
Bare soil	Control	7.302	7.024	9.472	8.086
Bare soil	Urine	7.721	6.957	9.514	8.102
Perennial RG	Control	7.177	6.823	9.49	7.946
Perennial RG	Urine	7.494	6.798	9.442	7.907
Italian RG	Control	7.152	6.862	9.51	7.967
Italian RG	Urine	7.392	6.764	9.445	7.897
	LSD (5%)	*0.1604*	*0.0659*	*0.0568*	*0.0762*

Table 4.6 Log-transformed means and LSDs for RNA copy numbers of ammonia-oxidising bacteria (AOB) and ammonia-oxidising archaea (AOA) in the soil after 61 days. Treatments (Control, and Urine at 700 kg N ha^{-1}) were applied in May 2015.

		AOB	AOA
Bare soil	**Control**	5.169	5.189
Bare soil	**Urine**	6.109	4.947
Perennial RG	**Control**	5.145	4.285
Perennial RG	**Urine**	5.699	4.727
Italian RG	**Control**	4.426	4.33
Italian RG	**Urine**	5.614	4.639
	LSD (5%)	*0.4668*	*0.876*

4.3.3 Soil chemistry

Soil NH_4^+-N concentrations increased following urine application (Figure 4.12; Table 4.7) and ranged from 203-322 mg NH_4^+-N kg soil^{-1} for the first three sampling occasions (days 1-30), and then rapidly declined to concentrations found in the non-urine (Control) perennial RG and Italian RG. The NH_4^+-N concentrations for bare soil-Urine remained higher and only decreased to 41 and 12 mg NH_4^+-N kg soil^{-1} on days 61 and 90, respectively. Perennial RG and Italian RG soil NH_4^+ concentrations were not significantly different throughout the experimental period. Log-transformed means and LSDs are shown in Table 4.7.

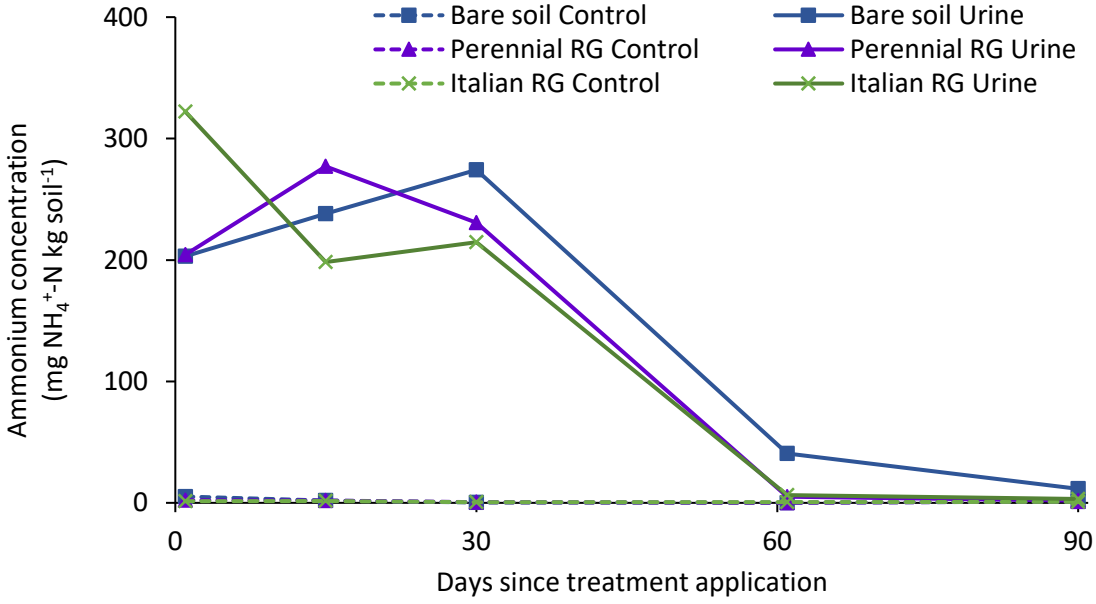


Figure 4.12 Back-transformed soil ammonium concentration (mg N kg soil^{-1}) after 1, 15, 30, 61, and 90 days following treatment application with or without urine (700 kg N ha^{-1}) in May 2015.

Table 4.7 Log-transformed means and LSDs for soil ammonium levels following treatment application with or without urine (700 kg N ha^{-1}) in May 2015.

Forage type	Treatment	Day 1	Day 15	Day 30	Day 61	Day 90
Bare soil	Control	0.792	0.465	0.161	0.041	0.313
Bare soil	Urine	2.310	2.378	2.440	1.620	1.099
Perennial RG	Control	0.507	0.470	0.190	0.047	0.316
Perennial RG	Urine	2.312	2.444	2.365	0.781	0.447
Italian RG	Control	0.366	0.400	0.189	0.217	0.238
Italian RG	Urine	2.509	2.300	2.334	0.865	0.624
LSD (5%)		*0.2386*				

Soil NO_3^--N concentrations were affected by forage type and urine treatments, and were higher for urine-treated soil than for the controls across all forage types ($P < 0.05$) from day 15 onwards. Bare soil-Urine had higher soil NO_3^--N concentrations than the perennial RG or Italian RG throughout the experimental period ($P < 0.05$) (Figure 4.13). Perennial RG soil NO_3^--N concentrations were not different to the bare soil-Control on days 30 and 90. Similarly, Italian RG-Urine NO_3^--N concentrations were not different to the bare soil-Control on days 15, 30, and 90 (Figure 4.13). Perennial RG and Italian RG soil NO_3^--N concentrations were not significantly different throughout the experimental period. Log-transformed means and LSDs are shown in Table 4.8. A significant linear relationship was found between soil NO_3^- concentration and AOB *amoA* gene abundance ($R^2 = 0.9145$) (Figure 4.14).

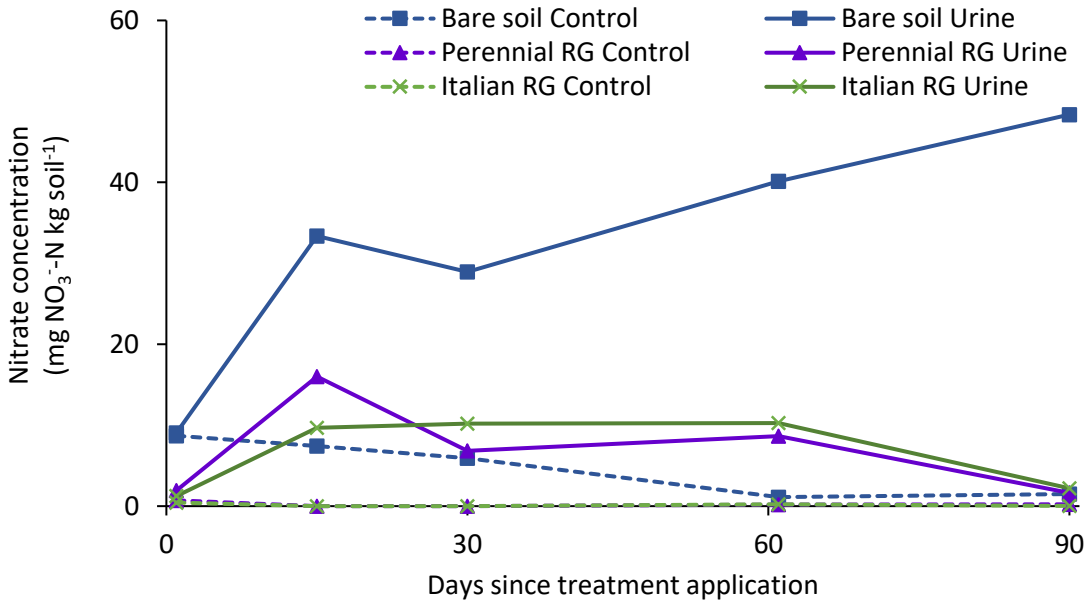


Figure 4.13 Back-transformed soil nitrate concentration (mg N kg soil^{-1}) after 1, 15, 30, 61, and 90 days following treatment application with or without urine (700 kg N ha^{-1}) in May 2015.

Table 4.8 Log-transformed means and LSDs for soil nitrate levels following treatment application with or without urine (700 kg N ha^{-1}) in May 2015.

Forage type	Treatment	Day 1	Day 15	Day 30	Day 61	Day 90
Bare soil	Control	0.987	0.925	0.841	0.326	0.396
Bare soil	Urine	1.002	1.536	1.476	1.614	1.693
Perennial RG	Control	0.234	0	0	0.076	0.090
Perennial RG	Urine	0.466	1.231	0.894	0.984	0.421
Italian RG	Control	0.159	0	0	0.088	0.014
Italian RG	Urine	0.350	1.029	1.049	1.052	0.506
LSD (5%)		*0.2261*				

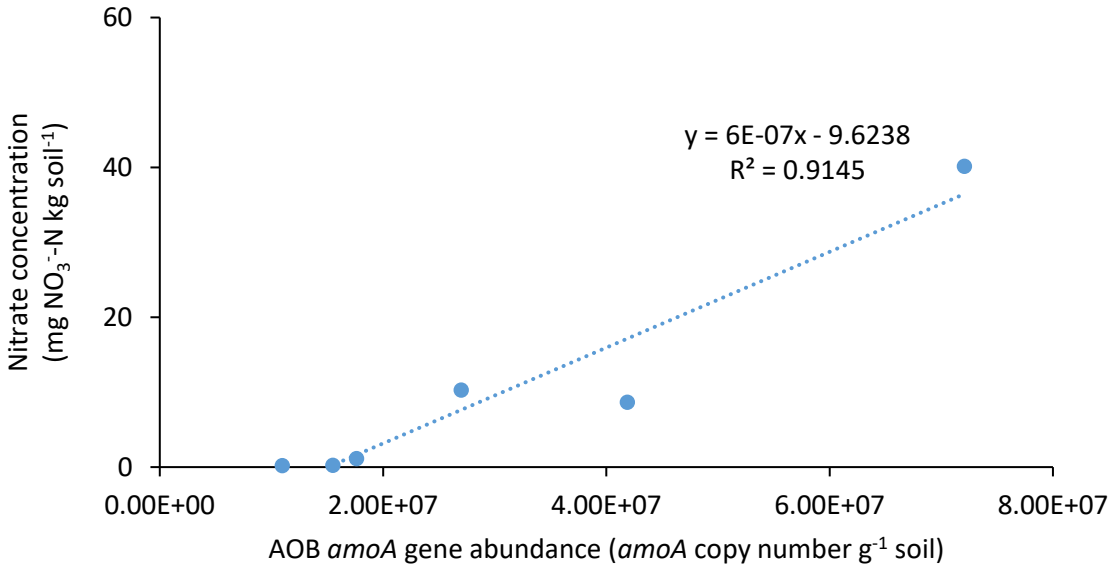


Figure 4.14 Relationship between nitrate-N concentration in soil and ammonia-oxidising bacteria DNA *amoA* gene abundance for all forages and treatments, 61 days after treatment application. Treatments (Control, and Urine at 700 kg N ha^{-1}) were applied in May 2015.

Soil gravimetric moisture content

Soil gravimetric moisture content (%) ranged from 11 to 29% throughout the experimental period (Figure 4.15). Moisture contents did not differ with treatment for the first three harvests, however by days 61 and 90 both of the urine-treated ryegrasses had lower soil moisture contents ($P < 0.05$), when compared with the other treatments.

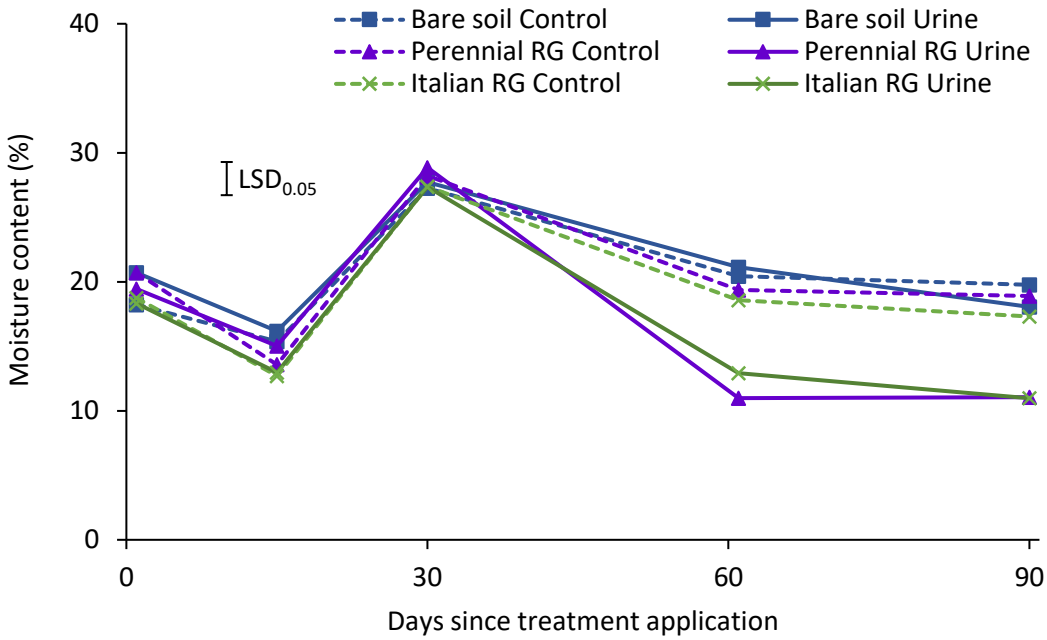


Figure 4.15 Soil gravimetric moisture content (%) after 1, 15, 30, 61, and 90 days following treatment application with or without urine (700 kg N ha^{-1}) in May 2015.

Soil pH

During the experimental period, soil pH ranged from 4.7 to 7.2 and was affected by both forage type (P < 0.001) and urine (P < 0.001) treatments, with a significant forage x treatment interaction (P = 0.015) (Figure 4.16). The main differences were observed between the urine-treated bare soil and both the ryegrasses. There was no significant difference in pH between perennial RG and Italian RG. The pH of the Control treatments remained constant for each forage type throughout the experimental period. The urine-treated pots within each forage type had a higher soil pH than the respective controls for the first 30 days. By days 61 and 90 the pH of the urine-treated pots had declined to be the same or lower than the corresponding controls within each forage type. During this time the urine-treated bare soil had the lowest pH at 4.7-5, when compared with a range of 5.8-6 for both of the urine-treated ryegrasses.

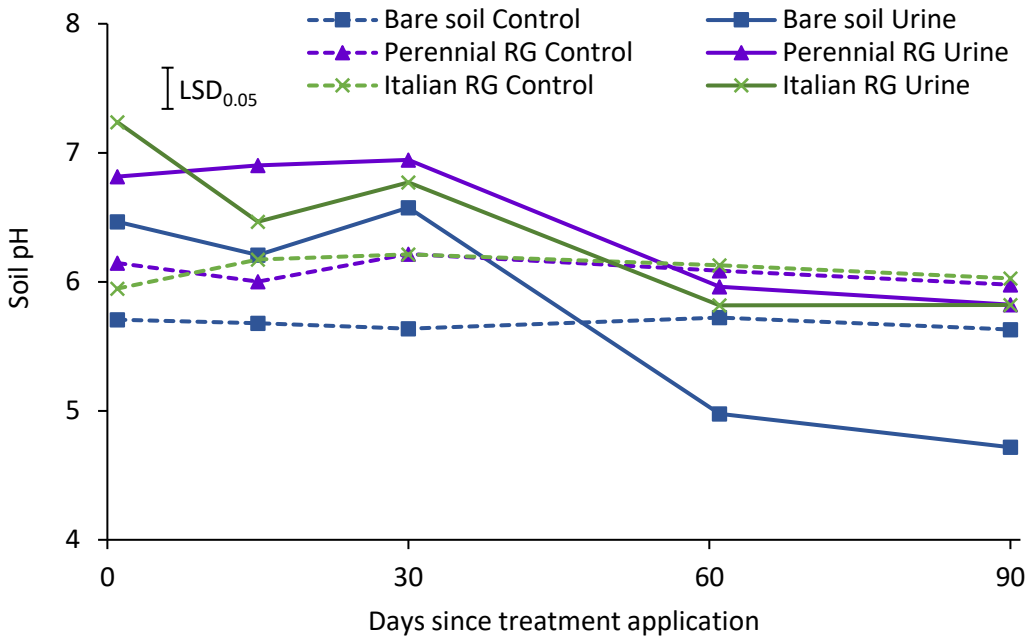


Figure 4.16 Soil pH after 1, 15, 30, 61, and 90 days following treatment application with or without urine (700 kg N ha^{-1}) in May 2015.

4.4 Discussion

4.4.1 Ammonia-oxidising communities

The observed increase in AOB *amoA* abundance following the application of urine, and the lack of any effect or reduced abundance of the AOA *amoA* gene (Figure 4.3, Figure 4.7), have been previously observed (Di *et al.*, 2010b; O'Callaghan *et al.*, 2010; Di & Cameron, 2011; Wakelin *et al.*, 2013; Di *et al.*, 2014; Hill *et al.*, 2014). This suggests that AOB and AOA prefer different soil N conditions to grow (Di *et al.*, 2010b) and that the application of urine could have an inhibitory effect on the AOA community in some soils (Di & Cameron, 2011). The significant relationship found between soil NO_3^--N and AOB *amoA* gene copy numbers (Figure 4.14) has also been shown by Di *et al.* (2010b), and is consistent with other studies where significant relationships have also been shown between *amoA* gene copy numbers of AOB in the soil and nitrate leaching loss (Di *et al.*, 2009a), and nitrification rate (Di *et al.*, 2009b). This led Di *et al.* (2009b) to suggest that nitrification was driven by bacteria rather than archaea in the high-nitrogen environments of grassland soils. Such conditions which would be present on New Zealand grazed systems were simulated in the current experiment. Many of the previous studies were conducted under laboratory conditions and, though there were plants present in some of the experiments (O'Callaghan *et al.*, 2010; Wakelin *et al.*, 2013; Hill *et al.*, 2014), none of these examined the effect of plants on AOB and AOA communities. In a previous study, Wakelin *et al.* (2009) showed pasture management affected soil microbial community structures and bacteria involved in N cycling, however pasture type (annual vs perennial) had a very minor effect. Similarly, Li *et al.* (2016) showed

AOB abundance was higher in the soil of grazed dairy and sheep farms than pine-forest soils. In contrast, AOA abundance was highest in the soil of the low N fertility grazed sheep farm in their study. They demonstrated the significant impact of land use on ammonia-oxidiser communities and this again reinforces that the AOB and AOA communities prefer different soil conditions for growth. This is discussed further in Section 4.4.3.

This experiment discovered a significant difference in AOB communities in the bare soil, compared with both ryegrasses. This indicated that the effect of plants vs no plants was greater than any subtle differences between ryegrass species. There was no significant difference between gene copy numbers in soil beneath perennial RG and Italian RG for any of the microbial communities measured (AOB, AOA, Bacteria 16S, Archaea 16S) (Figure 4.3, Figure 4.6, Figure 4.9, Figure 4.11). There was very little if any effect of forage type on non-target microbes (Bacteria 16S, Archaea 16S) in soil. It is unlikely that root architecture influenced the lower N leaching losses shown in Lysimeter Experiment 1 (Chapter 3), because recent studies, which have shown lower leaching losses from Italian RG, concluded that cool season growth (and thus N uptake) was more important than root architecture (Malcolm *et al.*, 2014; Malcolm *et al.*, 2015).

4.4.2 Soil nitrogen

Declining soil NH_4^+-N concentrations, observed in the urine treatments across all forage types, provide evidence of nitrification occurring between days 30 and 61 of the experimental period (Figure 4.12) which is consistent with other studies (Williams & Haynes, 2000; Di & Cameron, 2004; Guo *et al.*, 2014). The lack of any difference in soil NO_3^--N or NH_4^+-N concentrations between perennial RG and Italian RG (Figure 4.12 and Figure 4.13) demonstrates a lack of any biological nitrification inhibition by Italian RG. The lower NH_4^+-N concentrations in the two ryegrass treatments on days 61 and 90, compared with bare soil indicate that plant uptake of NH_4^+ occurred. The high soil NO_3^--N concentrations for the urine-treated bare soil, which continued to increase with time, demonstrates nitrification going to completion in the bare soil treatment, whereas the lower soil NO_3^--N concentrations for both ryegrasses, provide further evidence of N uptake by the plants (Figure 4.13). Because soil NH_4^+ and NO_3^- conditions were the same under both ryegrasses, it is unlikely that this caused the reduced N leaching observed under the Italian RG in Lysimeter Experiment 1 (Chapter 3). Other mechanisms by which NH_4^+ could have been lost (other than nitrification or N uptake, as previously discussed) include volatilisation to NH_3 or fixed by the soil via immobilisation (Jansson & Persson, 1982), these were not measured in the current study. However, pH for all treatments in the current study ranged from 4.7-7.2 throughout the experimental period meaning losses via NH_3 volatilisation are likely to have been minimal (du Plessis & Kroontje, 1964; Black *et al.*, 1985a; Black *et al.*, 1985b). Immobilisation is likely to have been similar in urine-treated pots due to the same soil and urine being used for all pots,

differences in soil chemistry and urine properties should have been minimised. Hence, treatment effects on these processes are unlikely and plant uptake is considered the main factor for the lower of NH_4^+ and NO_3^- concentrations observed under ryegrasses.

4.4.3 Physical parameters

As mentioned in the introduction, there are many soil physical parameters that could influence the nitrification process, and subsequently affect N leaching losses: especially soil texture, soil structure, temperature, moisture, aeration (O_2 and CO_2), pH, electrical conductivity, C:N ratio, cation exchange capacity (CEC), and organic matter (Subbarao *et al.*, 2006b). This experiment used soil collected from the same site for all of the pots, and therefore it is likely that parameters such as soil texture, and structure would have been similar between treatments. However, the plants may have influenced some other factors. Soil moisture content can have a marked effect on N leaching, as the higher the moisture, the more drainage which may occur. Similarly, Di *et al.* (2014) suggested that moisture has a major influence on ammonia-oxidising communities in urine-treated soils. They showed that ammonia-oxidisers are able to grow under very wet soil conditions, and that AOB population abundance was limited by the dry soil conditions at 60% of field capacity. Similar observations were made by O'Sullivan *et al.* (2013) where AOB tended to dominate soil samples collected during cool, wet periods whereas during hot, dry periods AOA dominated. No significant differences in moisture content occurred across all treatments for the first 30 days of this experiment (Figure 4.15). After this, an effect of plants became apparent. The bare soil pots remained the wettest, while the urine-treated ryegrasses grew more (visual observations), and in doing so would have extracted more water from the soil, drying it out more rapidly than the bare soil. There were no differences in soil moisture content between perennial RG, and Italian RG, and therefore, soil moisture is not likely to have caused the reduced N leaching shown in Lysimeter Experiment 1 (Chapter 3).

Soil pH is another factor which affects nitrification, with optimum soil pH being between 4.5 and 7.5 (Haynes, 1986a). Similarly, soil pH affects relative abundance of ammonia-oxidiser communities (Nicol *et al.*, 2008; Robinson *et al.*, 2014). Ammonia-oxidising bacteria and ammonia-oxidising archaea prefer different pH environments: a recent study showed AOB growth was favoured in the alkaline-treated soils (pH >6.5), whereas AOA growth was favoured in the acid-treated soil (pH ~5) (Robinson *et al.*, 2014), similarly AOA *amoA* gene and transcript abundance decreased with increasing pH, whereas AOB *amoA* transcripts increased with increasing pH (Nicol *et al.*, 2008). Liu *et al.* (2015) showed that AOA were more abundant than AOB in three Australian soils: a neutral soil (pH = 7), alkaline soil (pH = 8), and acid soil (pH = 4.6), treated with ammonium chloride (100 µg N g^{-1} soil). This suggested that in this case, AOA was more important for nitrification in these soils. Nitrification in the acid soil was mainly associated with the dynamics of AOA rather than AOB. Similarly, O'Sullivan *et al.* (2012) showed AOA

to be dominant in acidic (pH ($CaCl_2$) = 4.1-5.8) Australian soils, AOB *amoA* was not detected below pH 5. They determined that soil pH was a key driver of the AOA community structure and showed that AOA *amoA* gene abundance was inversely correlated with soil pH. The application of urine had the greatest effect on soil pH in the current experiment (Figure 4.16). The bare soil had a much lower pH than both the ryegrasses by days 61 and 90. This could be explained by nitrification, which is a net acidifying process (Black, 1992; Condon *et al.*, 2004), and Figure 4.13 showed a greater amount of soil NO_3^- in bare soil treatments than both the ryegrass treatments.

Soil temperature and aeration were not measured for pots but are likely to relate to soil moisture, which was not significantly different between perennial RG and Italian RG. These measurements should be considered for future experiments to eliminate these as causes for reduction in N leaching under Italian RG, compared with RGWC.

4.5 Conclusions

- Compared with bare soil, both ryegrasses reduced the abundance of ammonia-oxidisers, and the concentration of ammonium and nitrate in the soil. This is likely to be attributed to uptake of N by the plants in the ryegrass treatments.

- The findings of this experiment have failed to reject the null hypothesis. There was no significant difference in the abundance of AOB and AOA beneath perennial RG compared to Italian RG. Therefore, the main mechanism for Italian RG reducing N leaching from soil (observed in Lysimeter Experiment 1 (Chapter 3)) is likely to relate to N uptake over winter not any nitrification inhibitory effect.

Chapter 5

Lysimeter Experiment 2

5.1 Introduction

In New Zealand grazed systems, urine patches are the main source of NO_3^- leaching loss. Urine patches can cover 20-30% of a grazed field per year (Moir *et al.*, 2011) and the high N loading rates deposited in urine (average 613 kg N ha^{-1}, range 200-2000 kg N ha^{-1} for dairy cattle (Selbie *et al.*, 2015)) often exceed plant requirements. Nitrogen which is not taken up by plants is available to be leached from the soil in drainage (Cameron *et al.*, 2013) and can contaminate ground and surface waters.

One strategy to reduce the amount of urine-N which is leached is to reduce the loading of the urine patch by reducing the concentration of N which is excreted by the grazing animal. Recent studies have indicated that incorporating herbs such as chicory (*Cichorium intybus* L.) and plantain (*Plantago lanceolata* L.) in mixed swards can reduce the amount of N excreted in urine, while maintaining similar herbage yields to standard perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) (Woodward *et al.*, 2012; Totty *et al.*, 2013). Urinary-N concentrations of 0.26% and 0.34% (~260 and 340 kg N ha^{-1}) have been reported for mixtures containing plantain, compared with 0.62% and 0.57% (~620 and 570 kg N ha^{-1}) for perennial ryegrass-white clover (Woodward *et al.*, 2012; Totty *et al.*, 2013). Similarly, a modelling study which incorporated 20% and 50% diverse mixtures containing plantain and other species into a whole farm system predicted a 3.3-8.1% reduction in urinary-N excretion on an annual basis, compared with a standard perennial ryegrass-white clover system (Khaembah *et al.*, 2014). A review by Stewart (1996) has shown that plantain has highly palatable leaves and provides a mineral-rich forage for grazing animals. It is rapid to establish, drought tolerant, and grows on a range of different soil types, it is also tolerant of many pests and diseases. Plantain's deeper roots mean it can have a competitive advantage over shallower rooted grasses for water and nutrients (Stewart, 1996).

Nitrogen leaching losses were found to be 35% lower from Italian ryegrass (*Lolium multiflorum* Lam.) (Italian RG) than perennial ryegrass-white clover (RGWC) in Lysimeter Experiment 1 (Chapter 3) due to the ability of Italian RG to take up more urine-N during the winter period, when growth is often limited by cool temperatures. Since mixtures with plantain have previously been shown to reduce urine-N concentration, it is possible that a mixture containing both plantain and Italian ryegrass could have the potential to produce even greater reductions in nitrate leaching compared with perennial ryegrass-white clover.

Therefore, the objective of this experiment was to determine the N leaching loss, dry matter yield, and N uptake from the urine patch of an Italian ryegrass-plantain-white clover mix forage when compared with perennial ryegrass-white clover.

This experiment tested the following key hypotheses:

1. That an Italian ryegrass-plantain-white clover mixture would have a lower leaching loss than perennial ryegrass-white clover.
2. That cows grazing the Italian ryegrass-plantain-white clover mixture have lower urine-N excretion, compared with perennial ryegrass-white clover.
3. That the Italian ryegrass-plantain-white clover mixture would take up more N during the cool season than perennial ryegrass-white clover.

5.2 Methodology

5.2.1 Lysimeter collection and installation

In late November, early December 2014, 30 lysimeters (0.5 m diameter, 0.7 m deep) were collected from the Lincoln University Research Dairy Farm using the same methodology as described in Section 3.2.2. Fifteen of these lysimeters were taken from a field planted with a mixture of Italian ryegrass (*Lolium multiflorum* Lam.) cv. 'Tabu' (Agriseeds), plantain (*Plantago lanceolata* L.) cv. 'Tonic' (Agricom), and white clover (*Trifolium repens* L.) cv. 'Kopu II' (PGG Wrightson Seeds) (43°38'32.02" S, 172°27'44.94" E) (Italian-Plantain Mix) (Appendix A, Figure A 1). The other 15 lysimeters were from a field containing perennial ryegrass (*Lolium perenne* L.) and white clover (cv. 'Kopu II') (43°38'27.34" S, 172°27'43.96" E) (RGWC) (Appendix A, Figure A 1). The perennial ryegrass cultivar was 'Arrow' with AR1 endophyte (Agriseeds). The soil type is described in detail in Section 3.2.1. Soil from each collection site was tested before the start of the experiment to determine nutrient status and pH using the same methodology as described in Section 3.2.1 (Table 5.1). These results were used to inform fertiliser applications and ensure the fertility of each set of lysimeters was similar.

Field history

Both the RGWC and Italian-Plantain Mix fields were sown in March 2014 and had previously been rotationally grazed by Friesian-Jersey-cross cows. Irrigation had been applied with a centre-pivot irrigator. The botanical composition of the two fields are shown in Table 5.2.

Table 5.1 Soil test results of the fields where lysimeters were collected.

	Perennial ryegrass-white clover	Italian-Plantain Mix
pH	5.9	5.9
Olsen P (μg g^{-1})	25.3	31.6
Organic Matter (g kg^{-1})	45	47
Total C (g kg^{-1})	26.0	27.2
Total N (g kg^{-1})	2.2	2.3
Sulphate S (μg g^{-1})	6	12
CEC[1] ($cmol_c$ kg^{-1})	14	14
Exchangesable Ca^{2+} ($cmol_c$ kg^{-1})	7.7	7.5
Exchangeable Mg^{2+} ($cmol_c$ kg^{-1})	0.87	0.89
Exchangeable K^+ ($cmol_c$ kg^{-1})	0.51	0.68
Exchangeable Na^+ ($cmol_c$ kg^{-1})	0.24	0.25
BS[2] (%)	65	67.3

[1]Cation exchange capacity; [2]Base saturation

Table 5.2 Botanical composition of the two forages.

	RGWC	Italian-Plantain Mix
Perennial ryegrass (%)	48.4	
Italian ryegrass (%)		20.5
Plantain (%)		42.3
White clover (%)	25.7	27.7
Other (%)	3.8	0.6
Dead (%)	22.1	8.8

5.2.2 Treatments and experimental design

Lysimeter treatments are summarised in Table 5.3. There were six treatment combinations (two forage types, three urine rates), replicated five times. Lysimeters were installed in the trench facility in a straight line (Plate 5.1). The experiment was a randomised complete block design, with replicates as blocks. Within each replicate block, forage type and urine treatments were randomised using Genstat (16th Edition, VSN International Ltd.).

Table 5.3 Lysimeter treatments.

Treatment no.	Forage type	Treatment	Rate	Reps
T1	Perennial ryegrass + white clover (RGWC)	Control		5
T2	Perennial ryegrass + white clover (RGWC)	Urine Actual	664 kg N ha^{-1}	5
T3	Perennial ryegrass + white clover (RGWC)	Urine 700	700 kg N ha^{-1}	5
T4	Italian ryegrass + plantain + white clover mix (Italian-Plantain Mix)	Control		5
T5	Italian ryegrass + plantain + white clover mix (Italian-Plantain Mix)	Urine Actual	508 kg N ha^{-1}	5
T6	Italian ryegrass + plantain + white clover mix (Italian-Plantain Mix)	Urine 700	700 kg N ha^{-1}	5

Plate 5.1 Lysimeters installed in the trench facility at Lincoln University's Field Research Centre.

Treatment application

For the "Urine Actual" treatment, urine was collected from Friesian-Jersey-cross cows grazing each of the two different forage types. This was collected during the afternoon milking on 23 March 2015 and the morning milking on 24 March 2015 (after a three-day lead-in period). The total N concentration of the urine was determined on an Elementar Vario-Max CN Elemental Analyser (Elementar GmbH, Hanau, Germany). This was found to be 6.64 g N L^{-1} from cows grazing the RGWC forage, and 5.08 g N L^{-1} from cows grazing the Italian-Plantain Mix forage. Prior to application, urine was labelled with ^{15}N to an enrichment of 5 atom% by adding highly enriched ^{15}N urea (98 atom%; Cambridge Isotope Laboratories, Inc.). An appropriate volume of deionised water was added to the urine to account for this extra N added and return the urine-N concentration to the same as what it was at collection.

For the “Urine 700” treatment, urine was collected during the afternoon milking on 23 March 2015, the morning milking on 24 March 2015, and the afternoon milking 26 March 2015 from the cows grazing the RGWC forage. This urine was then enriched with ^{15}N, and unlabelled urea and glycine (9:1 ratio) were added so that the N concentration increased to 7 g N L^{-1} with a ^{15}N enrichment of 5 atom%. The glycine was used to represent the amino acid fraction of urine and better mimic the actions of real urine (Fraser *et al.*, 1994).

All of the urine was mixed thoroughly, 2 L was then measured out using a volumetric flask and applied to each appropriate lysimeter to simulate urine patches deposited by grazing dairy cows (Plate 5.2). Control plots received 2 L of water so that moisture inputs would remain consistent.

Plate 5.2 Application of urine treatments to lysimeters.

5.2.3 Lysimeter maintenance

Lysimeters were kept well-watered to keep the forages alive and prevent the soil from drying out and cracking over the summer period. Prior to treatment application, herbage was cut when at the 3 leaf stage or ~3000 kg DM ha^{-1} and discarded. From 4 February 2015 lysimeters were installed in a trench facility at Lincoln University’s Field Research Centre where they received irrigation using the same methodology described in Section 3.2.5.

Plate 5.3 The lysimeter sprinkler irrigation system in action.

Fertiliser applications

Lysimeters received 200 kg ha^{-1} of superphosphate (0:9:0:11) (18 kg P ha^{-1} and 22 kg S ha^{-1}) as maintenance fertiliser on 4 September 2015. Nitrogen was applied as urea to all lysimeters at a rate of 25 kg N ha^{-1} on 4 September 2015, 28 October 2015, 24 November 2015, 15 December 2015, 15 January 2016, 5 February 2016, 7 March 2016, and 7 April 2016.

Pest and weed control

Weeds were controlled within lysimeters by gentle hand weeding. Weeds which were removed following treatment application were left on the lysimeter surface. Yates Soil Insect Killer (50 g kg^{-1} diazinon in pellet form) was applied to lysimeters and the surrounding area on 26 February 2015 to control slugs.

5.2.4 Measurements

Leachate samples were collected and analysed (for NH_4^+-N, NO_3^--N, and ^{15}N enrichment) in the same manner as described in Section 3.2.6 except that preliminary leachate samples were only collected on two occasions. The RGWC and Italian-Plantain-Mix herbage was harvested once the ryegrass plant development had reached the 2-3 leaf stage (Figure 3.3) and yields were on average 3000 kg DM ha^{-1}. This was consistent with Lysimeter Experiment 1 (Chapter 3). Herbage was cut (Plate 5.4), dried, ground, and analysed for total N, ^{15}N enrichment and forage quality parameters using the same methodology as described Sections 3.2.7, 3.2.10, and 3.2.11 (forage morphology measurements were not carried out for this experiment).

Plate 5.4 Herbage is harvested using an electric hand piece, or hand clippers.

At the end of the experimental period, once the full mineral N (NO_3^--N + NH_4^+-N) leachate breakthrough curve had been completed, soil samples were taken from the lysimeters by destructive sampling at four depths: 0-15, 15-30, 30-45, and 45-65 cm. These were collected using a hand auger fitted with a bucket auger head (0.08 m diameter) to go down to each depth at two different locations in each lysimeter (Plate 5.5), and these were bulked for each lysimeter. Soils were analysed for inorganic N (NH_4^+-N and NO_3^--N), and gravimetric moisture content was determined in the same manner as in Section 3.2.8.

Plate 5.5 Soil sampling: a) using augers at the end of the experimental period, and b) the arrangement of the two different holes from which the soil samples were bulked.

5.2.5 Statistical analysis

Data were analysed using Genstat (18th Edition, VSN International Ltd.) by conducting an analysis of variance (ANOVA) as a 2 (forage type) x 3 (treatment) factorial with five blocks (randomised block design). Control data were excluded for determining treatment effects on N leaching loss by analysis of variance because the values were very low (<0.7 kg N ha^{-1}), as expected, and the treatments of

interest were forage type and urine application. Where significant effects were shown, the unrestricted LSD procedure (Saville, 1990) at either the 5% or 10% level was used to identify differences among means. Soil data were analysed for each soil depth.

5.3 Results

5.3.1 Climate data

During the experimental period (27 March 2015 to 5 September 2016), the mean daily air temperature ranged from a low of -0.4°C in July 2015 to a high of 24.8°C in February 2016 (Figure 5.1a). Similarly, daily mean soil temperature (10 cm depth) ranged from 1.8°C (July 2015) to 23.1°C (February 2016) (Figure 5.1a). Temperatures followed expected cyclical trends with warmer temperatures during summer and cooler temperatures during winter. Total water inputs for the 17-month experimental period were 1798 mm, including 804 mm of rainfall, and 994 mm of irrigation or simulated rainfall (Figure 5.1b). Monthly total water inputs are illustrated in Table 5.4.

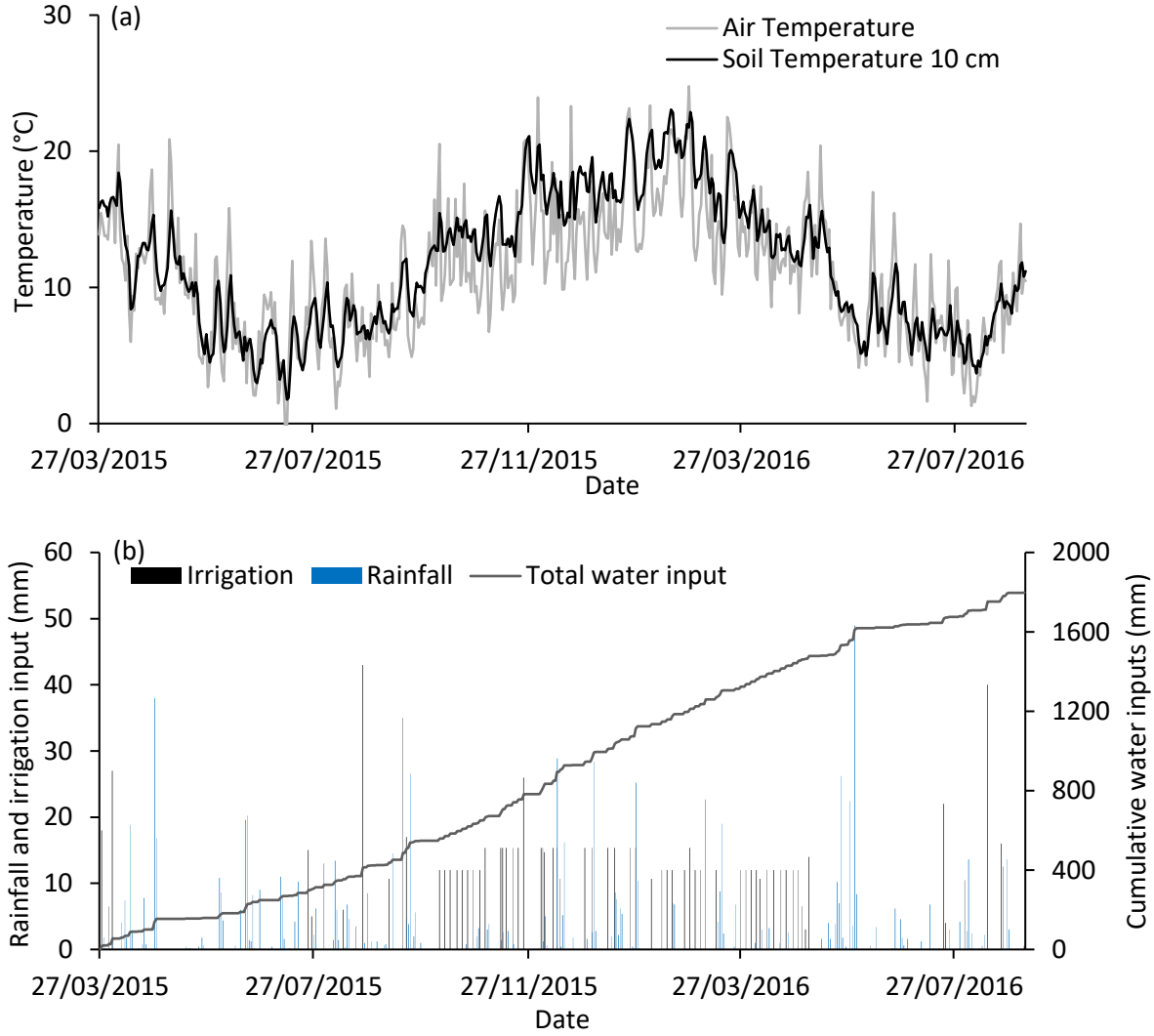


Figure 5.1 a) Average daily air temperature and soil temperature (at 10 cm), and b) daily and cumulative rainfall and irrigation (including simulated rainfall) water inputs (mm).

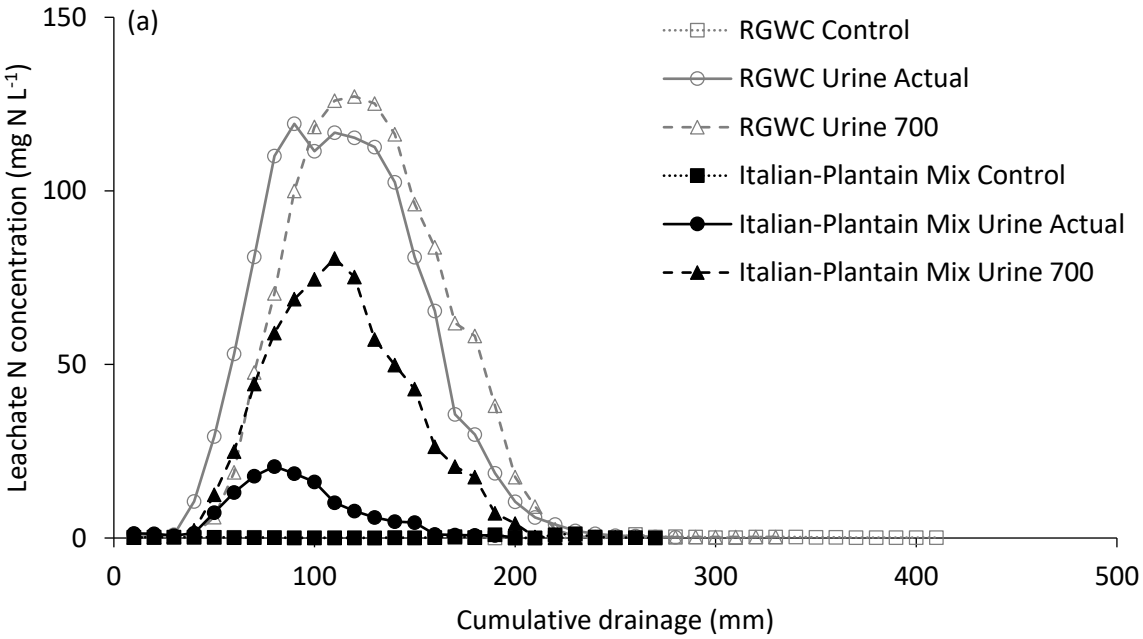
Table 5.4 Monthly total water inputs (mm) and average drainage (mm) for treatments. Perennial ryegrass and white clover (RGWC), and an Italian ryegrass, plantain, and white clover mixture (Italian-Plantain Mix) were treated in March 2015 with either: Control, Urine Actual (urine from cows grazing each forage type (664 kg N ha^{-1} for RGWC, and 508 kg N ha^{-1} for Italian-Plantain Mix)), or Urine 700 (urine at 700 kg N ha^{-1}).

		Year	2015						2016										
		Month	April	May	June	July	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	April	May	June	July	Aug
Total water inputs (mm)			135	4	90	64	111	124	103	132	163	179	88	124	118	155	28	42	118
Drainage (mm)	RGWC	Control	39	7	38	29	70	77	6	-	-	-	-	-	1	2	25	6	88
		Urine Actual	40	6	14	19	54	61	5	-	1	-	-	-	-	0	26	4	84
		Urine 700	43	7	19	17	49	59	4	-	-	-	-	-	1	1	15	5	77
	Italian-Plantain Mix	Control	34	8	28	19	51	66	7	-	1	0	-	-	1	2	10	2	72
		Urine Actual	32	6	1	5	36	46	4	-	-	0	-	-	1	8	6	3	78
		Urine 700	44	8	9	14	37	38	5	-	-	-	-	-	-	-	9	3	77
		LSD (5%)	15	5	10	10	14	13	2		1	1			2	10	11	4	14
	P-value	Forage	NS[1]	NS	<0.001	0.003	<0.001	<0.001	NS		NS	NS			NS	NS	<0.001	0.03	NS
		Treatment	NS	NS	<0.001	0.004	0.002	<0.001	0.014		NS	NS			NS	NS	NS	NS	NS
		F x T	NS	NS	NS	NS	NS	NS	NS		NS	NS			NS	NS	NS	NS	NS

[1]NS nonsignificant

5.3.2 Nitrogen leaching losses

A breakthrough curve of the leachate mineral N concentrations (NO_3^- + NH_4^+) shows that the concentrations increased with drainage to a peak and then declined to background levels (Figure 5.2a). Peak concentration values ranged from 21 to 127 mg N L^{-1} for urine-treated lysimeters (Figure 5.2a). Total mineral N leaching losses were 88.9% lower (P < 0.05) from Italian-Plantain Mix-Urine Actual (12.5 kg N ha^{-1}), when compared with RGWC-Urine Actual (112.7 kg N ha^{-1}) (Figure 5.2b). Similarly, N leaching losses were 45.5% lower (P < 0.1) from Italian-Plantain Mix-Urine 700 (61.8 kg N ha^{-1}), when compared with RGWC-Urine 700 (113.4 kg N ha^{-1}) (Figure 5.2b). There was no interaction (P = 0.232) between forage type and treatment (Urine Actual, Urine 700). Leaching losses from Control lysimeters were minimal (<0.7 kg N ha^{-1}). Total drainage volumes ranged from 284 mm (Italian-Plantain Mix-Urine 700) to 413 mm (RGWC-Control) over the experimental period. There was a difference in total drainage due to forage type (P < 0.001) and treatment (P = 0.003), however, there was no forage x treatment interaction (P = 0.647). The RGWC had the highest (P < 0.05) drainage volumes (average across treatments of 332.5 mm, compared with 256.4 mm for Italian-Plantain Mix). Averaged across forage type, drainage was highest (P < 0.05) in the Control treatment at 343.5 mm, compared with 270.5 mm (Urine 700), and 269.4 mm (Urine Actual). Monthly average drainage volumes for treatments are shown in Table 5.4, in general, there was less drainage from the Italian-Plantain Mix during the winter months (June-August).



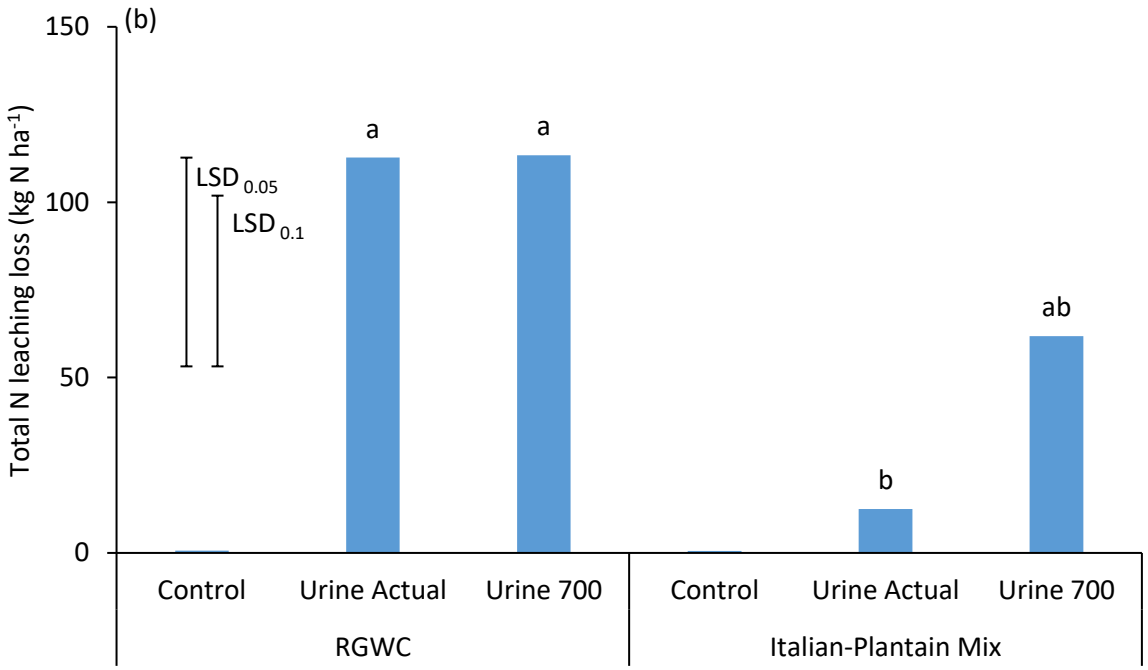


Figure 5.2 Mean mineral nitrogen leaching loss (NO_3^--N + NH_4^+-N): a) concentration (mg N L^{-1}) in leachate plotted against cumulative drainage, and b) total mineral nitrogen leaching loss (kg N ha^{-1}) from lysimeters for the experimental period: 27 March 2015 to 5 September 2016. Perennial ryegrass and white clover (RGWC), and an Italian ryegrass, plantain, and white clover mixture (Italian-Plantain Mix) were treated in March 2015 with either: Control, Urine Actual (urine from cows grazing each forage type (664 kg N ha^{-1} for RGWC, and 508 kg N ha^{-1} for Italian-Plantain Mix)), or Urine 700 (urine at 700 kg N ha^{-1}). Control means are plotted but not included in the statistical analysis. The error bars are least significant differences (LSD) at the 5% and 10% level. Bars with the same letter (a-b) are not significantly different at the 5% level.

5.3.3 Herbage yield and nitrogen uptake

Total herbage yield (t DM ha^{-1}) (Figure 5.3) and N uptake (kg N ha^{-1}) (Figure 5.4) harvested over the 17-month experimental period were both affected by treatment (Urine Actual, Urine 700) ($P < 0.001$), and there was no forage type x treatment interaction. There was no difference in herbage yield between the RGWC and the Italian-Plantain Mix forage. Herbage yield and N uptake were higher for both the urine-treatments, when compared with the respective controls (Figure 5.3 and Figure 5.4). Nitrogen uptake was affected by forage type ($P = 0.026$). Although there was no difference in the N uptake between the two forages for the Control and Urine Actual treatments, the RGWC-Urine 700 (744 kg N ha^{-1}) had a 14% greater N uptake than the Italian-Plantain Mix-Urine 700 treatment (656 kg N ha^{-1}) (Figure 5.4).

Cool season N uptake (kg N ha^{-1} d^{-1}) for urine-treated forage (Urine Actual, Urine 700) for the May harvest was greatest ($P < 0.05$) for the Italian-Plantain Mix herbage at 2.83-3.04 kg N ha^{-1} d^{-1}, compared

with corresponding urine-treated RGWC at 2.48-2.69 kg N ha^{-1} d^{-1} (Table 5.5). There was no significant effect of forage type on winter N uptake for the April, and August harvests.

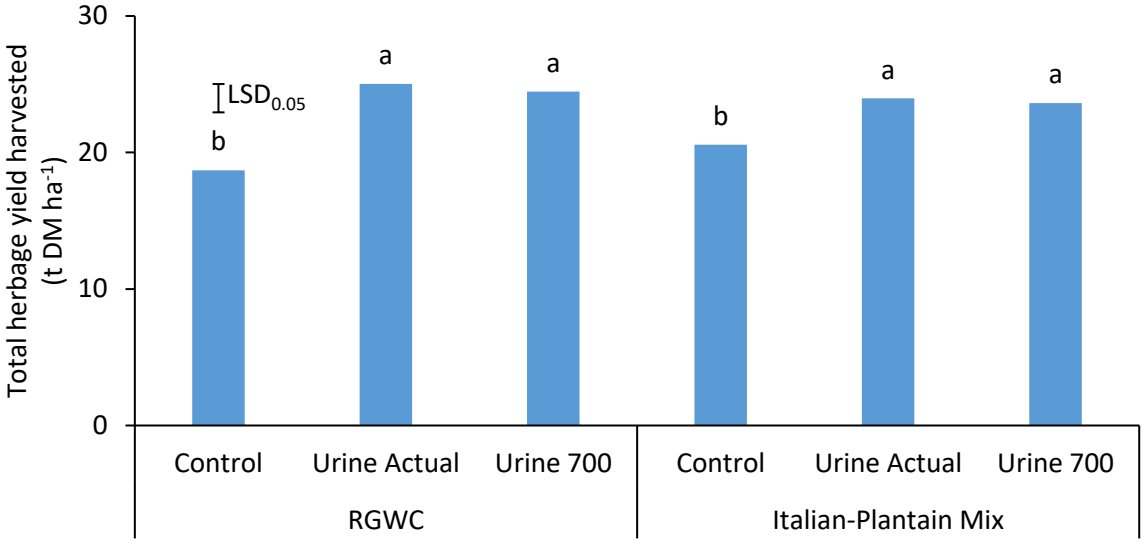


Figure 5.3 Total herbage dry matter yield harvested (t DM ha^{-1}) from lysimeters for the experimental period: 27 March 2015 to 5 September 2016. Perennial ryegrass and white clover (RGWC), and an Italian ryegrass, plantain, and white clover mixture (Italian-Plantain Mix) were treated in March 2015 with either: Control, Urine Actual (urine from cows grazing each forage type (664 kg N ha^{-1} for RGWC, and 508 kg N ha^{-1} for Italian-Plantain Mix)), or Urine 700 (urine at 700 kg N ha^{-1}). Bars with the same letter (a-b) are not significantly different at the 5% level.

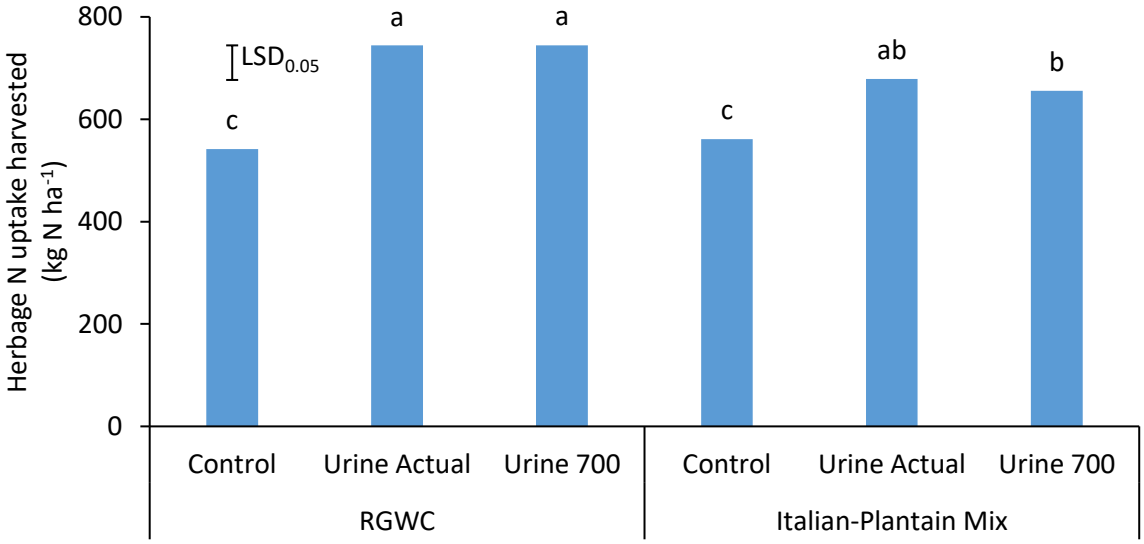


Figure 5.4 Nitrogen uptake harvested (kg N ha^{-1}) from lysimeters for the experimental period: 27 March 2015 to 5 September 2016. Perennial ryegrass and white clover (RGWC), and an Italian ryegrass, plantain, and white clover mixture (Italian-Plantain Mix) were treated in March 2015 with either: Control, Urine Actual (urine from cows grazing each forage type (664 kg N ha^{-1} for RGWC, and 508 kg N ha^{-1} for Italian-Plantain Mix)), or Urine 700 (urine at 700 kg N ha^{-1}). Bars with the same letter (a-c) are not different at the 5% level.

Table 5.5 Nitrogen uptake harvested (kg N ha^{-1} d^{-1}) for winter herbage (DM yield x N% ÷ rotation length). Perennial ryegrass and white clover (RGWC), and Italian ryegrass, plantain and white clover (Italian-Plantain Mix) were treated in March 2015 with either: Control, Urine Actual (urine from cows grazing each forage type (664 kg N ha^{-1} for RGWC, and 508 kg N ha^{-1} for Italian-Plantain Mix)), or Urine 700 (urine at 700 kg N ha^{-1}). Means in each column with the same letter (a-d) are not significantly different at the 5% level.

Forage Type	Treatment	*Harvest 21/04/2015 (kg N ha^{-1} d^{-1})*	*Harvest 25/05/2015 (kg N ha^{-1} d^{-1})*	*Harvest 11/08/2015 (kg N ha^{-1} d^{-1})*
RGWC	Control	1.44^{c}	0.90^{d}	0.15^{b}
RGWC	Urine Actual	3.31^{a}	2.69^{bc}	0.94^{a}
RGWC	Urine 700	3.07^{a}	2.48^{c}	0.91^{a}
Italian-Plantain Mix	Control	1.43^{c}	0.96^{d}	0.24^{b}
Italian-Plantain Mix	Urine Actual	3.46^{a}	3.04^{a}	0.90^{a}
Italian-Plantain Mix	Urine 700	2.36^{b}	2.83^{ab}	1.03^{a}
P value	Forage Type	0.345	0.011	0.49
P value	Treatment	<0.001	<0.001	<0.001
P value	FxT	0.188	0.338	0.68
$LSD_{0.05}$		0.7063	0.3342	0.2774

5.3.4 ^{15}N balance

Recovery of ^{15}N within the herbage and leachate pools was affected by forage type ($P < 0.05$), and there was an effect of treatment for the herbage pool. Herbage-^{15}N recoveries were highest for the Italian-Plantain Mix-Urine Actual treatment (45.6%), compared with the other treatments (33.3-35.5%) (Table 5.6, Figure 5.5). Leachate-^{15}N recovery was significantly lower for the Italian-Plantain Mix-Urine Actual treatment (1.5%), compared with 6-11.8% for all other treatments (Table 5.6, Figure 5.6). Herbage-N derived from urine varied between forage type with the N in Italian-Plantain Mix-Urine 700 having the greatest amount derived from urine-N (39.1%). Leachate-N derived from urine was not affected by forage type or urine treatment (Table 5.7).

Table 5.6 Recovery (%) of the ^{15}N applied with the urine, in the herbage and leachate fractions (n = 5). Numbers in each column with the same letter (a-b) are not significantly different at the 5% level.

Forage Type	Treatment	Herbage	Leachate
RGWC	**Urine Actual**	34.3 ^{b} ± 4.5[1]	11.8 ^{a} ± 9.1
	Urine 700	33.3 ^{b} ± 5.6	11.8 ^{a} ± 12.4
Italian-Plantain Mix	**Urine Actual**	45.6 ^{a} ± 3.9	1.5 ^{b} ± 5.4
	Urine 700	35.5 ^{b} ± 4.7	6.0 ^{ab} ± 4.1
***P* Value[2]**	**Forage**	**	**
	Treatment	*	NS
	FxT	NS	NS
	$LSD_{0.05}$	6.031	6.73

[1]± 95% Confidence interval;

[2]NS nonsignificant, * significant at the 0.05 probability level, ** significant at the 0.01 probability level.

Table 5.7 Percentage (%) of the N in herbage, and leachate which was derived from the applied urine (n = 5). Numbers in each column with the same letter (a-b) are not significantly different at the 5% level.

Forage Type	Treatment	Herbage	Leachate
RGWC	**Urine Actual**	31.7 ^{b} ± 4.3[1]	67.3 ^{ab} ± 3.6
	Urine 700	32.2 ^{b} ± 4.4	73.3 ^{a} ± 1.6
Italian-Plantain Mix	**Urine Actual**	35.4 ^{ab} ± 1.2	53.0 ^{b} ± 14.7
	Urine 700	39.1 ^{a} ± 4.9	63.0 ^{ab} ± 13.1
***P* Value[2]**	**Forage**	*	NS
	Treatment	NS	NS
	FxT	NS	NS
	$LSD_{0.05}$	6.131	17.54

[1]± 95% Confidence interval;

[2]NS nonsignificant, * significant at the 0.05 probability level.

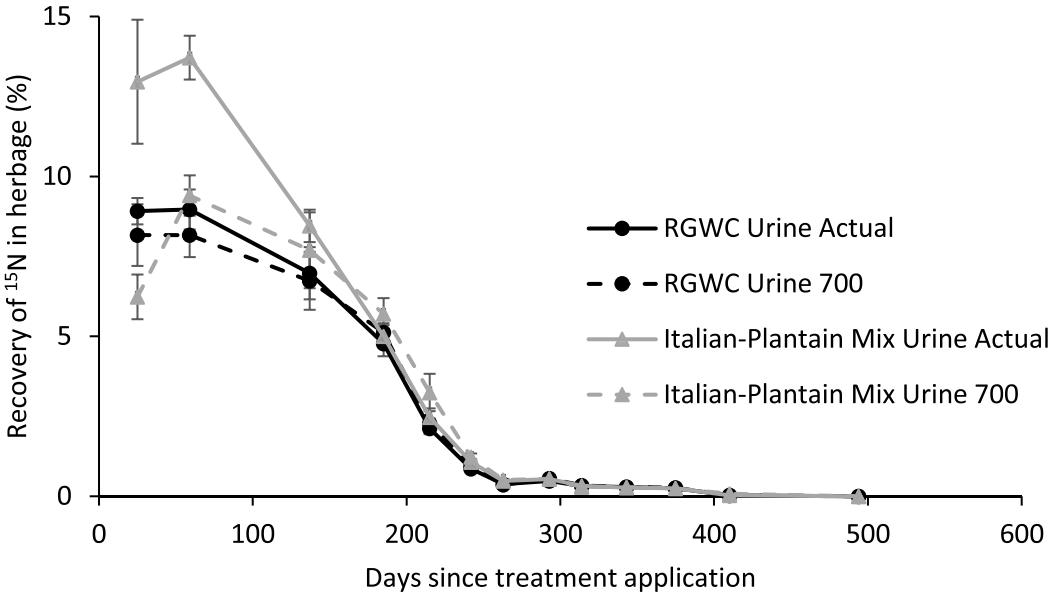


Figure 5.5 Herbage-^{15}N recovery (%) throughout the experimental period: 27 March 2015 to 5 September 2016. Perennial ryegrass and white clover (RGWC) and Italian ryegrass, plantain, and white clover mix (Italian-Plantain Mix) were treated in March 2015 with either: Control, Urine Actual (urine from cows grazing each forage type (664 kg N ha^{-1} for RGWC, and 508 kg N ha^{-1} for Italian-Plantain Mix)), or Urine 700 (urine at 700 kg N ha^{-1}). Error bars are standard error of the mean (n = 5).

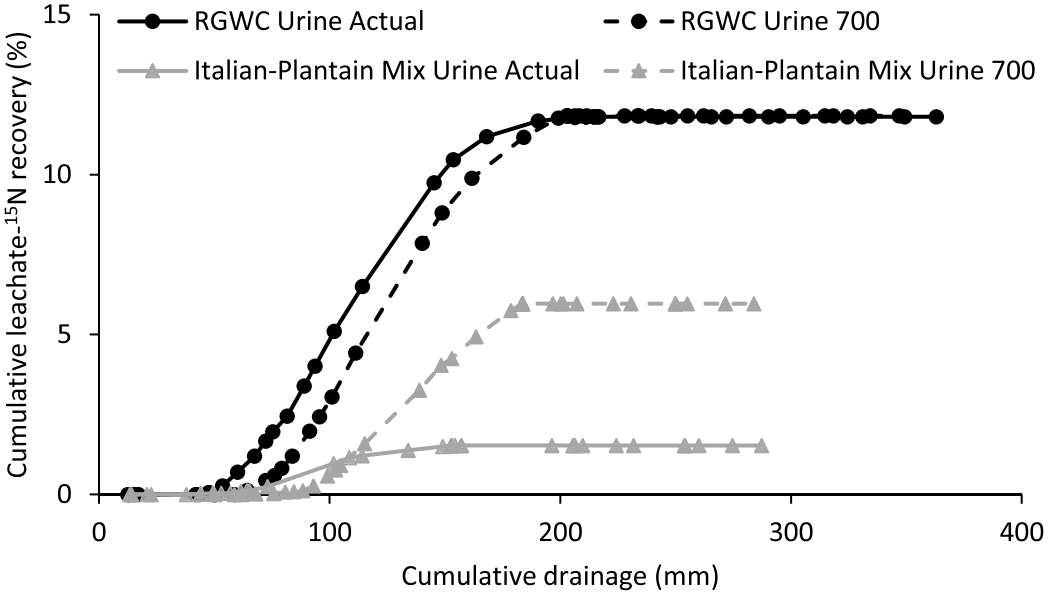


Figure 5.6 Mean cumulative recovery of leachate-^{15}N (%) against drainage over the experimental period: 27 March 2015 to 5 September 2016. Perennial ryegrass and white clover (RGWC) and Italian ryegrass, plantain, and white clover mix (Italian-Plantain Mix) were treated in March 2015 with either: Control, Urine Actual (urine from cows grazing each forage type (664 kg N ha^{-1} for RGWC, and 508 kg N ha^{-1} for Italian-Plantain Mix)), or Urine 700 (urine at 700 kg N ha^{-1}).

5.3.5 Soil

Soil NH_4^+-N concentrations were very low (<0.03 mg NH_4^+-N kg $soil^{-1}$) and were not affected by forage type or urine treatment at all four depths measured (0-65 cm). Soil NO_3^--N concentrations were also very low (<0.5 mg NO_3^--N kg $soil^{-1}$) and were affected by forage type at the 0-15 cm depth (P = 0.023), but were not significantly different for the other depths down to 65 cm. Soil NO_3^--N concentrations were highest for Italian-Plantain Mix at the 0-15 cm depth (P < 0.05).

5.4 Discussion

5.4.1 Effects of forage type on N leaching loss

The 89% lower N leaching losses from the Italian-Plantain Mix-Urine Actual (12.5 kg N ha^{-1}), compared with RGWC-Urine Actual (112.7 kg N ha^{-1}) is a highly significant finding which reinforces that manipulation of the forages which make up the diet of grazing animals is a promising management tool which can help to reduce N leaching losses. These treatments took into account the urine-N excreted by cows grazing each forage type. The excretion of urine-N by grazing cows was 23.5% lower from Italian-Plantain Mix (508 kg N ha^{-1}) than RGWC (664 kg N ha^{-1}), this appears to have had a marked effect on the N leaching losses and ability of the herbage to take up urine-N. There was also less total drainage from Italian-Plantain Mix lysimeters (Section 5.3.2), which was mostly due to the lower drainage volumes during the winter months (June-August) (Table 5.4). This suggests greater water uptake by the Italian-Plantain Mix forage during this time. It is important to note because total N leaching losses are derived from both the concentration of N in the leachate but also the volume of drainage which occurred. There was no difference in the dry matter (DM) yield between urine-treated (Urine Actual, Urine 700) forages (RGWC, Italian-Plantain Mix). Similarly, there was no difference in total N uptake between the Italian-Plantain Mix-Urine Actual and Italian-Plantain Mix-Urine 700 or RGWC-Urine Actual. However, the ^{15}N recovery data show a significantly higher herbage-^{15}N recovery for the Italian-Plantain Mix-Urine Actual treatment, compared to all the other urine-treated forages. It is likely that the lower rate of urine-N allowed better capture of the urine-N by the plants following urine application (Figure 5.5). This indicates that the proportion of N applied (e.g. as urine) which is taken up by plants may be more important than total N uptake, for reducing N leaching losses. A modelling study by Li *et al.* (2012) supports the current experimental findings of lower N leaching losses due to lower urine-N deposition, they stated that "actions that promote urine depositions with a low N concentration would significantly reduce N leaching". They also provide a comparison between N leaching from the urine patch to the that from the field and explore the effects of variation in urine volume and urine-N concentration on N leaching.

This reduction in urine-N excretion observed in urine collected from the two forages in the current experiment is consistent with other studies (Al-Mamun *et al.*, 2008b; Woodward *et al.*, 2012; Totty *et al.*, 2013; Edwards *et al.*, 2015). Similarly, in a recent study, Box *et al.* (2016) measured urine-N excretion from a 100% plantain forage, perennial ryegrass-white clover, and a 50:50 plantain:perennial ryegrass-white clover treatment. They measured lower urine-N concentrations than in the current study with 5.4 g N L^{-1} for the perennial ryegrass-white clover forage, a 56% reduction for the plantain forage (2.4 g N L^{-1}), and a 33% reduction for the 50:50 treatment (3.6 g N L^{-1}). Lower NH_3, urea, and creatinine in the urine of cows grazing plantain was also shown. In contrast to this, studies with heifers have shown no significant difference in autumn urine-N concentration between heifers fed a standard RGWC diet, compared with one containing plantain (Carr, 2015; Cheng *et al.*, 2015; Cheng *et al.*, 2017). Cheng *et al.* (2017) also found no significant difference in daily urine-N excretion (g day^{-1}) for heifers grazing 100% plantain compared with 100% perennial ryegrass-white clover. However, spring urine-N concentrations and urine-N excretion were lower for heifers grazing plantain (2.9 g kg^{-1} and 87 g day^{-1} $heifer^{-1}$, respectively), compared with perennial ryegrass-white clover (4.8 g kg^{-1} and 116 g day^{-1} $heifer^{-1}$, respectively) (Cheng *et al.*, 2017). The authors suggested that the reduced urine-N may have reflected the lower N intake of the heifers (224 vs 348 g day^{-1} $heifer^{-1}$ for plantain and perennial ryegrass-white clover, respectively) but also said that higher water intake due to lower dry matter of the plantain (spring DM content was 129 vs 193 g DM kg^{-1} and DM intake was 7.2 vs 10 kg DM day^{-1} $heifer^{-1}$ for plantain and perennial ryegrass-white clover, respectively) may have led to increased urine volume, with diluted N concentration (Cheng *et al.*, 2017). Similarly, Box *et al.* (2016) suggested one explanation for the lower N excretion in their study could be due to differences in urine volume, of which there was some evidence of in the reduced urine creatinine levels for cows grazing plantain forages, they said this may have been caused increased water intake due to the lower DM% of plantain observed in their study, or by plant secondary metabolites. Similarly, aucubin and acteoside (both plant secondary metabolites of plantain) have recently been shown to reduce NH_3 production in the rumen *in vitro* and have the potential to reduce the N losses in the urine of ruminant animals (Navarrete *et al.*, 2016).

As well as better N capture by the plants, another mechanism by which the plants could be influencing the amount of N which is leached could be by biological nitrification inhibition, this is a strategy whereby plants produce allelochemicals that suppress soil nitrification (Subbarao *et al.*, 2006b). In a recent review, Gardiner *et al.* (2016) discussed the potential for forage diet manipulation in New Zealand pasture ecosystems to mitigate another N cycling process: N_2O emissions. They suggested that plant secondary metabolites could play a role in these processes due to their known antimicrobial properties. In particular, they reviewed the literature on aucubin, which is an iridoid glycoside found in plantain and showed it to have promising potential to inhibit N_2O production, but further research

was needed. Aucubin is a glycoside of an unstable aglycone: aucubigenin which has a broad range of potential biological activity (Bartholomaeus & Ahokas, 1995). Aucubin has been found to have activity against a range of bacteria and fungi (Davini *et al.*, 1986). Aucubin concentrations in plantain were shown to be highest in mid-autumn when air temperatures were ~20°C (Tamura, 2002) and highest in younger plant tissue (Pankoke *et al.*, 2013). Similarly, from spring to mid-autumn, aucubin levels were shown to vary with cultivar and were 2.1-4.8% in Grasslands Lancelot and 1.0-2.7% in Ceres Tonic (Tamura & Nishibe, 2002). Plant secondary metabolites such as aucubin were not measured in the current study.

Soil incubation experiments have shown inhibition of soil N mineralisation when aucubin, plantain plant material, and plantain leaf extracts were incorporated with the soil (Dietz *et al.*, 2013). The suggested mechanism for this was that aucubin affects the soil N mineralisation actively via its reactive aglycone and/or passively via the influence of its hydrolysation product glucose on soil biota. A negative relationship between the concentration of iridoid glycosides (aucubin and catalpol) and soil-N concentrations was shown (Dietz *et al.*, 2013). The addition of aucubin also seemed to suppress nitrification. The suggested mechanisms for this were that the aucubigenin was mainly responsible and that it could act as an ammonia monooxygenase (AMO) substrate-like molecule that encroaches on the active site of AMO, but the authors recommended further investigations be carried out (Dietz *et al.*, 2013). Similarly, nitrification activities and mineral N concentrations were shown to be almost zero, and numbers of nitrifying bacteria reduced 200-fold in the presence of plantain plants, compared with no plants (N source was ammonium fertiliser, total of 320 mg NH_4^+-N per pot, not urine) (Verhagen *et al.*, 1995). However, these authors suggest that the main mechanism for this was that the plantain was more competitive for the limited levels of NH_4^+ than the nitrifying bacteria (*Nitrosomonas europaea*), and that the involvement of allelochemicals originating from plant roots was unlikely (Verhagen *et al.*, 1995). Similarly, in the Microbiology Pot Experiment (Chapter 4), the presence of plants reduced the abundance of ammonia-oxidising bacteria involved in nitrification as well as the levels of NH_4^+ in soil which was attributed to plant N uptake. However, aucubigenin has been shown to inhibit cytochrome P-450 (Bartholomaeus & Ahokas, 1995), which was previously shown to inhibit ammonia oxidation (Hooper & Terry, 1973).

It was not within the scope of this study to investigate the mechanism for the reduced urine-N excretion by grazing animals, but this study does illustrate that if forage diet manipulation does reduce urine-N excretion, this can significantly reduce N leaching losses from urine patches of grazed dairy systems.

There are many other benefits of incorporating plantain into forage mixtures: it can produce similar or greater DM yields (Malcolm, 2013; Nobilly *et al.*, 2013; Totty *et al.*, 2013; Woodward *et al.*, 2013; Macfarlane *et al.*, 2014), and milk production (Minnee *et al.*, 2012; Woodward *et al.*, 2012; Totty *et al.*, 2013; Woodward *et al.*, 2013; Edwards *et al.*, 2015; Box *et al.*, 2016) to perennial ryegrass-white clover. Forages containing plantain can provide good heifer growth rates (de Clifford *et al.*, 2014; Handcock *et al.*, 2015), and comparable or improved lamb growth rates, compared with perennial ryegrass-white clover (Brown *et al.*, 2014; Macfarlane *et al.*, 2014; Morris & Kenyon, 2014). In a review of the suitability of plantain as a pasture species, Stewart (1996) described that plantain is rapid to establish, its leaves are highly palatable to grazing animals (Stewart, 1996), and provide a mineral rich forage (Pirhofer-Walzl *et al.*, 2011). Plantain is drought tolerant, can grow on a wide range of agricultural soils, and is tolerant of many common diseases and pests (Marak *et al.*, 2000, 2002; Biere *et al.*, 2004). Stewart (1996) stated that "the presence of antimicrobial compounds capable of affecting the rumen fermentation process is likely to have important implications for rumen efficiency, mineral nutrition of ruminants, animal performance, milk composition, bloat and animal health."

5.4.2 Comparison with findings of Lysimeter Experiment 1 (Chapter 3)

The current experiment was conducted to advance knowledge and understanding beyond the findings of Lysimeter Experiment 1 (Chapter 3), thus it is interesting to discuss the differences in results found between these two experiments. Nitrogen leaching losses in the current experiment were lower than those measured in Lysimeter Experiment 1 (Chapter 3). There was a 700 kg N ha^{-1} urine treatment in both experiments which provides a direct comparison. Total N leaching loss from RGWC-Urine 700 was 113.4 kg N ha^{-1}, whereas in Lysimeter Experiment 1 (Chapter 3) this was much higher at 186.2 kg N ha^{-1}. The Italian-Plantain Mix-Urine 700 leaching losses (61.8 kg N ha^{-1}) were much lower than the Italian RG (132.4 kg N ha^{-1}) in Lysimeter Experiment 1 (Chapter 3). Air and soil temperatures were similar to Lysimeter Experiment 1 (Chapter 3) throughout the experimental period, however temperatures during the cool season (May-Sept) following urine application were around 0.84-0.85°C lower in the current experiment (based on daily mean temperatures). More rain fell in the current experimental period (804 mm, compared with 713 mm for Lysimeter Experiment 1 (Chapter 3)). Total water inputs were lower in the current experiment (1798 mm, compared with 1965 mm for Lysimeter Experiment 1 (Chapter 3)), due to less irrigation being applied. Drainage volumes were lower for the current experiment (284-413 mm vs 321-502 mm for Lysimeter Experiment 1 (Chapter 3)) likely as a result of the lower water inputs throughout the experimental period. Recovery of ^{15}N (applied with urine application) in leachate from RGWC-Urine 700 was lower in the current experiment than in the Lysimeter Experiment 1 (Chapter 3) (11.8 vs 23.7%, respectively). Similarly, there was less ^{15}N recovered in leachate from the Italian-Plantain Mix-Urine 700 treatment than the Italian RG in Lysimeter Experiment 1 (Chapter 3) (6 vs 16.8%, respectively). Unlike Lysimeter Experiment 1 (Chapter

3), the reduced recovery of ^{15}N in leachate did not correspond to an increase in herbage uptake of ^{15}N applied with the urine for the Urine 700 treatment. The RGWC-Urine 700 and Italian-Plantain Mix-Urine 700 treatments had similar herbage-^{15}N recoveries (33.3 and 35.5%, respectively). However, in the current experiment herbage-^{15}N recoveries were significantly higher for the Italian-Plantain Mix-Urine Actual treatment (45.6%), than the RGWC-Urine Actual treatment (34.3%), and leachate-^{15}N recoveries were even lower for the Italian-Plantain Mix-Urine Actual treatment (1.5%).

Herbage DM yields for RGWC-Urine 700 were similar in both experiments (~24 t DM ha^{-1}), however the Italian-Plantain Mix-Urine 700 (23.6 t DM ha^{-1}) had a higher DM yield in the current experiment than the Italian RG (20 t DM ha^{-1}) in Lysimeter Experiment 1 (Chapter 3). Total N uptake for the RGWC-Urine 700 was lower in the current experiment than in Lysimeter Experiment 1 (Chapter 3) (744.5 vs 811 kg N ha^{-1}, respectively). This could be attributed to the slightly cooler temperatures during the cool season following urine application in the current experiment. The Italian-Plantain Mix-Urine 700 took up more N than the Italian RG treatment in Lysimeter Experiment 1 (Chapter 3) (655.9 vs 629 kg N ha^{-1}, respectively). In Lysimeter Experiment 1 (Chapter 3), the lower leaching losses from Italian RG were mainly attributed to the higher uptake of N during the winter period by this forage type. This was reinforced in the Microbiology Pot Experiment (Chapter 4), where no differences in ammonia-oxidisers were found between soil beneath perennial RG and Italian RG, indicating the likely cause of the lower leaching loss to relate to winter N uptake. The ^{15}N data also reinforced this theory. In the current experiment, N uptake was only higher for the Italian-Plantain Mix in the month of May, in April it was lower than the RGWC-Urine 700, and there was no significant difference in August. It is difficult to compare the winter N uptake values of the current experiment with those from Lysimeter Experiment 1 (Chapter 3) due to the difference in harvest dates and the average daily air and soil temperatures between harvests. In the current experiment, air temperatures were on average 13.1, 11.3, and 6.5°C during the growth periods of the 21/4/2015, 25/5/2015, and 11/8/2015 harvests, respectively. Soil temperatures were 14.2, 11.2, and 6.2°C, respectively. For Lysimeter Experiment 1 (Chapter 3) air temperatures were on average 8.7, and 7.8°C during the growth periods of the 23/6/2014, and 7/8/2014 harvests, respectively. Soil temperatures were 8.6, and 7.1°C, respectively. Recovery of ^{15}N in herbage of RGWC-Urine 700 was lower in the current experiment than in the Lysimeter Experiment 1 (Chapter 3) (33.3 vs 40.1%, respectively). Similarly, the Italian-Plantain Mix-Urine 700 treatment also recovered less ^{15}N than the Italian RG in Lysimeter Experiment 1 (Chapter 3) (35.5 vs 49.5%, respectively).

The N_2O emissions from the current experiment are published in Di *et al.* (2016), these were higher for the current experiment than Lysimeter Experiment 1 (Chapter 3), which may explain where some of the N, which was not leached (but also does not appear to have been take up in herbage), may have

gone. Cumulative N_2O emissions of 20.9, 27.7, 14.8, and 33.24 kg N_2O-N ha^{-1} were found for RGWC-Urine Actual, RGWC-Urine 700, Italian-Plantain Mix-Urine Actual, and Italian-Plantain Mix-Urine 700, respectively. Whereas values for RGWC-Urine in Lysimeter Experiment 1 (Chapter 3) were 10.9 kg N_2O-N ha^{-1}, and for Italian RG-Urine were 9.57 kg N_2O-N ha^{-1}. Other possible sources of this urine-N include the soil, although soil ammonium and nitrate levels were low <0.5 mg N kg $soil^{-1}$. It may have been lost as NH_3 via volatilisation. This is possible as there was no rain on the day of urine application, however, 18 mm of irrigation occurred the following day, <10 hours after urine application. If rainfall or irrigation occurs within 48 hours of urine application, it is possible to significantly reduce the amount of NH_3 loss (Black *et al.*, 1987). If NH_3 volatilisation did occur, this could have been a significant source of the unaccounted for N as a review by Selbie *et al.* (2015) estimated that ~13% of N applied as urine could be lost via ammonia volatilisation. Similarly, in Table 2.1 a summary of ^{15}N studies showed that 0.7-50% of urine-N could be lost via ammonia volatilisation. Another possible source of the unaccounted for N is denitrification to N_2. Losses of N_2 have been measured at 26% of urine-N applied for perennial ryegrass on a sandy loam soil (Selbie, 2014). Total soil N, NH_3, and N_2 emissions were not measured in the current experiment.

5.5 Conclusions

- Nitrogen leaching losses were 88.9% and 45.5% lower from the Italian-Plantain Mix than RGWC for the Urine Actual, and the Urine 700 treatments, respectively. This was attributed partially to the Italian-Plantain Mix having higher winter activity and ability to take up N during the cool winter period than RGWC and partly to the lower concentration of urine-N collected from cows grazing the Italian-Plantain Mix which was accounted for in the Urine Actual treatment and had the greatest reduction in leaching.

- The Italian ryegrass-plantain-white clover mix has shown to be a promising forage mixture which can reduce excretion of urine-N and significantly reduce the amount of N lost by leaching, while maintaining the same dry matter yields as perennial ryegrass-white clover.

Chapter 6

Gibberellic Acid Experiment

6.1 Introduction

Nutrient management is an important environmental issue facing the agricultural industry today. The loss of N, which is a valuable nutrient, from the farm system represents a loss of potential productivity and is therefore of economic significance to the farm business. The N that is lost can also have a detrimental effect on the environment through reducing water quality and via emission of greenhouse gases which contribute to global warming. In grazed agricultural systems in New Zealand, it is the animal urine patches which are the major source of N leaching loss, due to the N loading greatly exceeding plant N requirements. In 2014, the New Zealand Government issued a National Policy Statement on Freshwater Management (Ministry for the Environment, 2014). Under this policy, Regional Councils must ensure that freshwater quality standards are met in rivers and lakes within their region. Thus Regional Councils are currently putting together Land and Water Plans which will help to meet their obligations under this policy. Nitrogen is specifically mentioned in regional plans because in many areas, N loads in some water bodies are higher than that which is considered sustainable. Limits on N leaching loss are being imposed in many catchments already via the Land and Water Plans. For consents to be granted, many farmers will be required to reduce the amount of N leaching from their property below their current levels. Thus there is an urgent need for options which farmers can use to mitigate N leaching losses.

In Lysimeter Experiment 1 (Chapter 3), it was hypothesised that gibberellic acid (GA) could be used as a mitigation option to reduce N leaching losses (due to its ability to increase pasture dry matter (DM) production in the shoulders of the growing season when cool temperatures limit plant growth (Matthew *et al.*, 2009)). It has even been proposed by Parsons *et al.* (2013) as a potential N leaching mitigation tool as they thought it could enhance plant uptake of N. As discussed in Sections 2.2.5 and 3.1, the literature regarding how GA application affects the N or crude protein (CP) levels in the plant is conflicting. To the best of the author's knowledge Lysimeter Experiment 1 (Chapter 3) is the first time that the effect of GA on N leaching losses has been measured.

Lysimeter Experiment 1 (Chapter 3) showed that the application of GA to a 700 kg N ha^{-1} urine patch on three different forages (perennial ryegrass-white clover, Italian ryegrass, and lucerne) had no effect on N leaching loss, herbage DM yield, or N uptake during the 17-month experimental period. However, as discussed in Section 3.4.3, forage treated with GA and lower rates of N (e.g. 20-50 kg N ha^{-1}) as

fertiliser have shown an increased DM response in addition to that of the fertiliser alone (Morgan & Mees, 1958; van Rossum *et al.*, 2013; Ghani *et al.*, 2014; Zaman *et al.*, 2014). Thus it is possible that at lower rates of urine-N deposition a response to GA may occur. Similarly, if an increase in DM caused by GA occurs, this could reduce the use of N fertilisers and therefore reduce the inputs of N cycling through a farm system (Whitehead & Edwards, 2015).

Therefore, the objective of this experiment was to determine the N leaching loss, DM yield, and N uptake response of perennial ryegrass-white clover to an application of gibberellic acid over a range of different urine-N rates (0-700 kg N ha^{-1}).

The experiment tested the following key hypothesis:

1. That the application of GA to perennial ryegrass-white clover reduces N leaching from urine patches in autumn, but that there is a maximum urine-N rate above which this effect is negligible.

6.2 Methodology

6.2.1 Lysimeter collection and installation

In February 2016, 56 lysimeters (0.18 m diameter, 0.5 m deep) were collected from the Lincoln University Research Dairy Farm (Appendix A, Figure A 1) using a similar methodology to that described in Section 3.2.2. The differences were that the casings for this experiment were made of PVC pipe, and the lysimeters were able to be removed from the soil profile using a spade, and so did not require the use of a cutting plate (Plate 6.1). Due to the smaller size of these lysimeters, only the bottom 20-30 mm of soil was replaced with gravel (Plate 6.1e). A plastic cap with a nozzle in the centre was fixed onto the bottom of the casing using silicon sealant (Plate 6.1f). Plastic tubing was connected to the base of each lysimeter and fed into a 5 L container for leachate collection. The lysimeters were then installed into four wooden boxes (1.21 m^2, 0.55 m high) (Plate 6.1g). Holes were drilled in the base of the wooden boxes to allow the tubing to go through and soil was filled in around the lysimeters (Plate 6.1h) to provide insulation similar to conditions in the field. The wooden boxes were placed on top of two heavy duty wooden pallets to allow drainage to flow downwards into collection containers below (Plate 6.3).

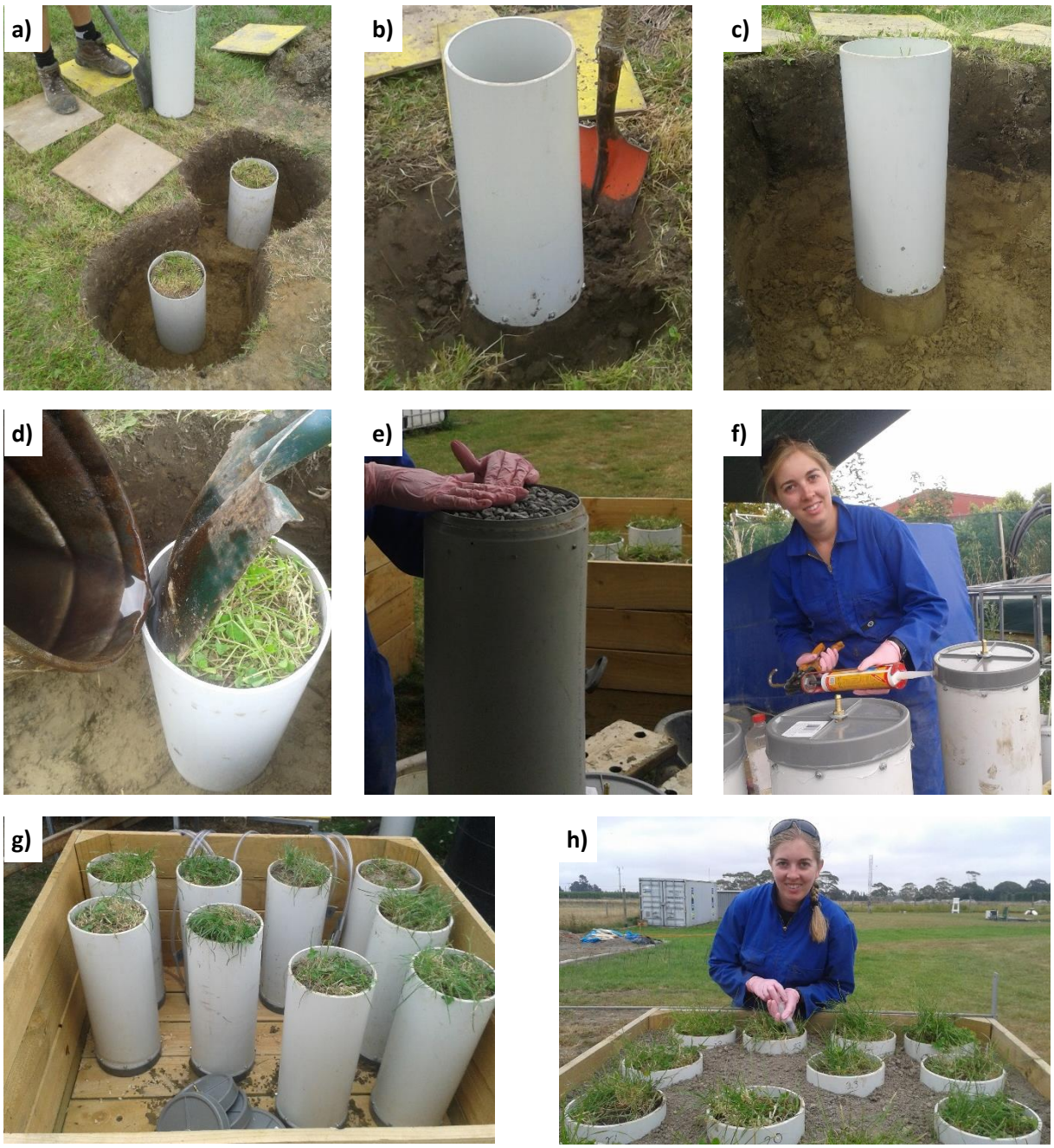


Plate 6.1 Lysimeter collection where a-c) shows lysimeters being dug down into the soil profile, d) the edges are sealed with Petroleum jelly, e) the bottom 20-30 mm of soil is replaced with gravel, f) silicon is used to seal and fix the base caps onto the casing, g) the lysimeters are installed into wooden boxes, and h) soil is filled in around the lysimeters and Petroleum jelly topped up around the edges.

6.2.2 Experiment description and preparation

The forage in the field where the lysimeters were collected from was a perennial ryegrass (*Lolium perenne* L., cv. 'One50' with AR37 endophyte) and white clover (*Trifolium repens* L., cv. 'Kopu II') mixture. This field was sprayed with glyphosate, cultivated, power harrowed and rolled on 12 March 2014. The perennial ryegrass was sown at 20 kg seed ha^{-1} and the white clover was sown at 3 kg seed

ha^{-1} using a Flexiseeder® on 20 March 2014. Cattle were excluded from the area from the time of sowing until lysimeter collection. Irrigation had been applied by a rotorainer with applications occurring 3-4 times a week with a total application of 550 mm for the 6-month period between October and March. The plots received 200 kg ha^{-1} of 20% potash sulphur super fertiliser (equivalent of 13 kg P ha^{-1}, 20 kg S ha^{-1}, and 33 kg K ha^{-1}) on 26 September 2014 and 3 t ha^{-1} of lime on 1 October 2014. The soil type at the collection site was a Paparua sandy loam, this is described in detail in Section 3.2.1, and is pictured in Plate 6.2. Soil tests were conducted to determine nutrient status and pH of the soil prior to the experiment starting (Table 6.1).

Table 6.1 Soil test (0-7.5 cm) results of the lysimeter collection site.

	Perennial ryegrass-white clover
pH	7.1
Olsen P (µg g^{-1})	20.8
Organic Matter (g kg^{-1})	3.6
Total C (g kg^{-1})	2.09
Total N (g kg^{-1})	0.19
Sulphate S (µg g^{-1})	26
CEC[1] ($cmol_c$ kg^{-1})	12
Exchangeable Ca^{2+} ($cmol_c$ kg^{-1})	10
Exchangeable Mg^{2+} ($cmol_c$ kg^{-1})	0.69
Exchangeable K^+ ($cmol_c$ kg^{-1})	0.35
Exchangeable Na^+ ($cmol_c$ kg^{-1})	0.12
BS[2] (%)	95.7

[1]Cation exchange capacity; [2]Base saturation

Plate 6.2 Paparua sandy loam soil at the lysimeter collection site for this experiment on the Lincoln University Research Dairy Farm.

6.2.3 Treatments and experimental design

Experimental design

The experiment was a randomised complete block design with four replicate blocks (the wooden boxes) (Plate 6.3). Treatments described in Table 6.2 were randomly allocated within replicate blocks using Genstat (16th Edition, VSN International Ltd.).

Plate 6.3 Lysimeter experimental setup: a) showing all four wooden boxes with collection containers, b) one replicate wooden box with collection containers, and c) the layout of lysimeters within the replicate boxes.

Treatment application

The day before treatment application, herbage was harvested to ~50 mm height using hand clippers. Fresh cow urine (>20 L) was collected from Friesian-Jersey-cross (KiwiCross^{TM}) cows during the afternoon milking at the Lincoln University Research Dairy Farm. Samples of this urine were analysed overnight for total N concentration using an Elementar Vario-Max CN Elemental Analyser (Elementar GmbH, Hanau, Germany). The urine collected had a concentration of 4.8 g N L^{-1}. On 20 April 2016, the day after urine collection, urine was measured into seven different containers. For the urine application rates of 25, 50, 100, 200, and 400 kg N ha^{-1}, urine was diluted with deionised water to decrease the urine-N concentration. For the 700 kg N ha^{-1} urine application rate, urea and glycine (9:1 ratio) were added to increase the urine-N concentration. Each of these were mixed thoroughly and 254 mL was measured out using a measuring cylinder and applied to each appropriate lysimeter. The 0 kg N ha^{-1} treatment received an equivalent volume of water to ensure that moisture inputs were consistent.

On 21 April 2016, a gibberellic acid solution (8 g GA ha^{-1}) was prepared by serial dilution: 1 g of ProGibb®SG (containing 40% gibberellic acid, Valent BioSciences Corporation, IL, USA, marketed by Nufarm Ltd., New Zealand) was dissolved with deionised water and 2.5 mL of surfactant (Spreadwet 1000, active constituent: 1000 g L^{-1} alkoxylated alcohols, SST NZ Ltd.) and made up to volume with deionised water in a 0.1 L volumetric flask. A 5 mL aliquot was transferred to another 0.1 L volumetric flask and made up to volume with deionised water. Then a 10 mL aliquot from the second flask was transferred to a 0.05 L volumetric flask and made up to volume with deionised water. Lysimeters received 0.51 mL of this gibberellic acid solution, which was applied evenly to the surface of appropriate lysimeters using an airbrush sprayer (Plate 3.7). Treatment shields (200 mm high) were used to prevent spray drift. Control (non-GA treated) lysimeters received an application of 0.51 mL of surfactant-only solution using the same preparation and application technique.

Table 6.2 Lysimeter treatments.

Treatment no.	GA[1] Treatment	Urine-N Treatment (kg N ha^{-1})	Replication
T1	0 (surfactant only)	0	4
T2	0 (surfactant only)	25	4
T3	0 (surfactant only)	50	4
T4	0 (surfactant only)	100	4
T5	0 (surfactant only)	200	4
T6	0 (surfactant only)	400	4
T7	0 (surfactant only)	700	4
T8	GA (8 g GA ha^{-1}) (+surfactant)	0	4
T9	GA (8 g GA ha^{-1}) (+surfactant)	25	4
T10	GA (8 g GA ha^{-1}) (+surfactant)	50	4
T11	GA (8 g GA ha^{-1}) (+surfactant)	100	4
T12	GA (8 g GA ha^{-1}) (+surfactant)	200	4
T13	GA (8 g GA ha^{-1}) (+surfactant)	400	4
T14	GA (8 g GA ha^{-1}) (+surfactant)	700	4

[1]Gibberellic acid, 8 g GA ha^{-1} is the commercial rate which is used and previous studies have shown this to give a DM response (Matthew *et al.*, 2009; Jiang *et al.*, 2011; Ball *et al.*, 2012)

6.2.4 Lysimeter maintenance

Fertiliser applications and weed control

Prior to the experiment starting, lysimeters received an application of 556 kg superphosphate ha^{-1} (equivalent to 50 kg P ha^{-1} and 61 kg S ha^{-1}) based on initial soil fertility results. Dock (*Rumex* spp.) was controlled by applying Harmony® (active ingredient: 75% thifensulfuron-methyl in the form of a water dispersible granule at a rate of 1 g L^{-1}). Other weeds were controlled by targeted glyphosate application to the weed herbage only. Weeds outside the lysimeters were controlled by hand weeding. Ammonium sulphate (NH_4SO_4; 21:0:0:24) was applied to the lysimeters on 19 September 2016 at a rate of 30 kg N ha^{-1} to simulate typical dairy farm practice.

Rainfall and irrigation simulation

Rainfall was monitored using a rain gauge fixed to a fence post nearby (Plate 6.3a). Irrigation and simulated rainfall was applied to the experiment by a hand held sprinkler system. This system had a spray nozzle (Tee Jet FL-5VC) and timer. The same system as described in Section 3.2.5 was used to decide when to apply irrigation or simulated rainfall. Irrigation was applied in March-April 2016, and simulated rainfall from May 2016.

6.2.5 Measurements

Leachate samples were collected and analysed (for NH_4^+-N, and NO_3^--N) in the same manner as described in Section 3.2.6 except that no preliminary leachate samples were collected. Herbage was harvested once the ryegrass plant development had reached the 2-3 leaf stage (Figure 3.3) and yields were on average 3000 kg DM ha^{-1}. This was consistent with Lysimeter Experiment 1 (Chapter 3). Herbage was cut, dried, ground, and analysed for total N using the same methodology as described Section 3.2.7 (herbage morphology and NIRS measurements were not carried out for this experiment).

6.2.6 Statistical analysis

Data were analysed using Genstat (18th Edition, VSN International Ltd.) by conducting an analysis of variance (ANOVA) as a 2 (GA treatment) x 7 (urine treatment) factorial with four blocks (randomised block design). For the urine treatment factor, polynomial contrasts were included in the ANOVA. The variables N leaching loss, herbage DM yield, and N uptake were log-transformed to achieve homogeneity of variance. Where significant effects were shown, the unrestricted LSD procedure (Saville, 1990) at the 5% level was used to identify differences among means. Where log transformations were used for statistical analysis, log means are displayed in tables with LSDs, and for graphs, data were back-transformed using anti-log to be more easily compared with other studies.

6.3 Results

6.3.1 Climate data

During the experimental period (20 April 2016 to 17 October 2016), the mean daily air temperature ranged from a low of 1.3°C in August to a high of 20.4°C in May (Figure 6.1a). Similarly, mean daily soil temperature (10 cm depth) ranged from 3.7°C (August) to 16.1°C (May) (Figure 6.1a). For 11 weeks following the application of GA, soil temperatures were within the recommended range of 5°C-16°C for use of GA on pasture (Matthew *et al.*, 2009). Total water inputs for the 6-month experimental period were 683 mm, which included 294 mm of rainfall and 389 mm of irrigation or simulated rainfall (Figure 6.1b). Monthly total water inputs are illustrated in Table 6.3.

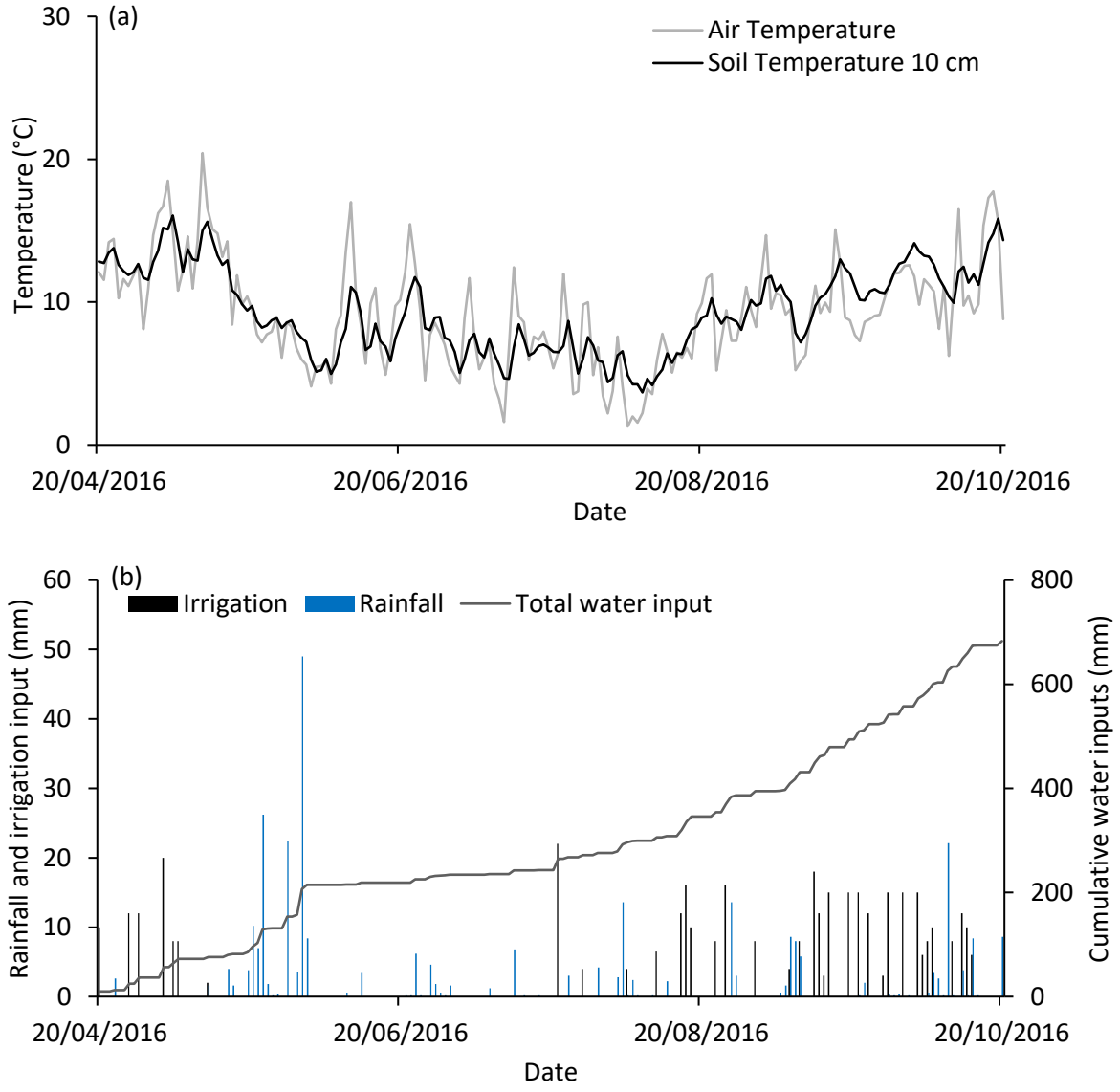


Figure 6.1 a) Average daily air temperature and soil temperature (at 10 cm), and b) daily and cumulative rainfall and irrigation (including simulated rainfall) water inputs (mm).

Table 6.3 Monthly total water inputs (mm) of rainfall and irrigation (including simulated rainfall) for the experimental period: 20 April 2016 to 17 October 2016.

Month	April	May	June	July	Aug	Sept	Oct
Total water inputs (mm)	37	170	28	42	119	163	125

6.3.2 Nitrogen leaching losses

A breakthrough curve of the leachate mineral N concentrations (NO_3^--N + NH_4^+-N) shows that for urine-N rates >200 kg N ha^{-1} the mineral N concentrations increased with drainage to a peak and then declined to background levels (Figure 6.2). Peak concentration values were 70-74 mg N L^{-1} for the 700 kg N ha^{-1} urine rate, 12-15 mg N L^{-1} for the 400 kg N ha^{-1} urine rate, and <4 mg N L^{-1} for urine rates below 200 kg N ha^{-1} (Figure 6.2).

A significant linear relationship was found between the urine-N rate and log-transformed total N leaching loss ($P < 0.001$) (quadratic relationship $P = 0.075$). The application of GA had no significant effect on N leaching loss ($P = 0.685$). Total N leaching loss at the end of the experimental period was very minimal (<2 kg N ha^{-1}) for urine-N applications rates of 0 to 200 kg N ha^{-1} (Figure 6.3). At the higher urine-N application rates of 400 and 700 kg N ha^{-1} N leaching losses were significantly higher with values of 4.5-5.6 kg N ha^{-1} and 27-35 kg N ha^{-1}, respectively. Log-transformed means and LSDs are shown in Table 6.4. The analysis of the data was complex, so three approaches were used to fit a line to data in Figure 6.3. The relationship between the urine-N application rate and the back-transformed total N leaching loss (averaged across GA treatments) was described by the following quadratic equation (Figure 6.3):

$$y = 0.001x^2 - 0.0273x + 1.3717 \ (R^2 = 0.9955)$$

The back-transformed linear relationship between the urine-N application rate and the log-transformed total N leaching loss (averaged across GA treatments) was described by the following exponential equation (Figure 6.3):

$$y = 10^{(-0.4659+0.002693x)}$$

The back-transformed quadratic relationship between the urine-N application rate and the log-transformed total N leaching loss (averaged across GA treatments) was described by the following exponential equation (Figure 6.3):

$$y = 10^{(-0.3703+0.001193x+0.000002197x^2)}$$

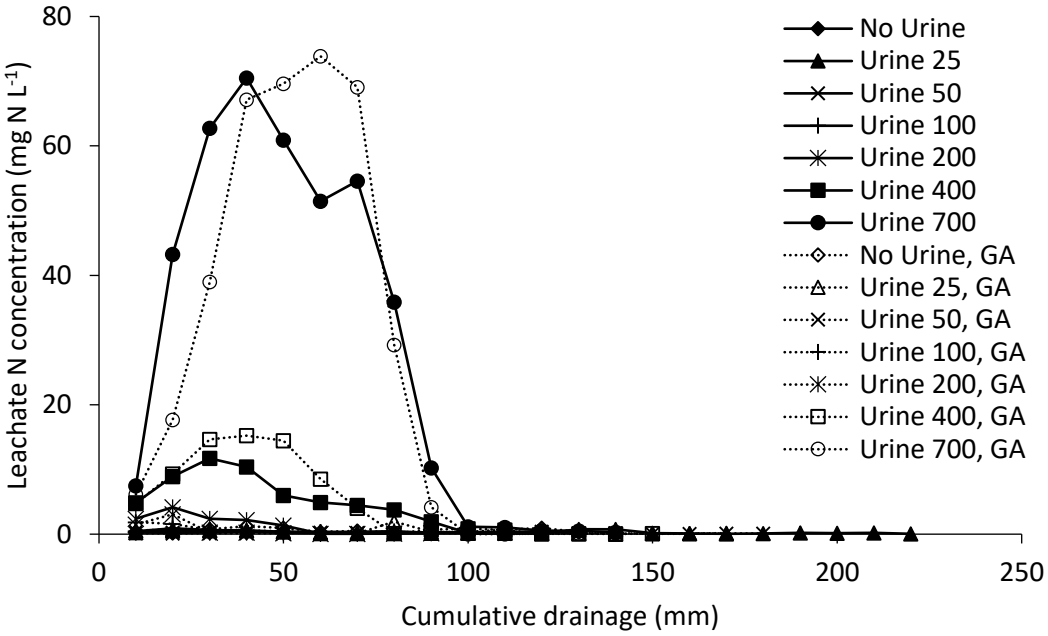


Figure 6.2 Mean mineral nitrogen (NO_3^--N + NH_4^+-N) concentration (mg N L^{-1}) in leachate plotted against cumulative drainage (mm) for the experimental period (20 April 2016 to 17 October 2016). Lysimeters received urine applications ranging from 0 to 700 kg N ha^{-1} and were treated with or without application of gibberellic acid (8 g GA ha^{-1}) on 21 April 2016.

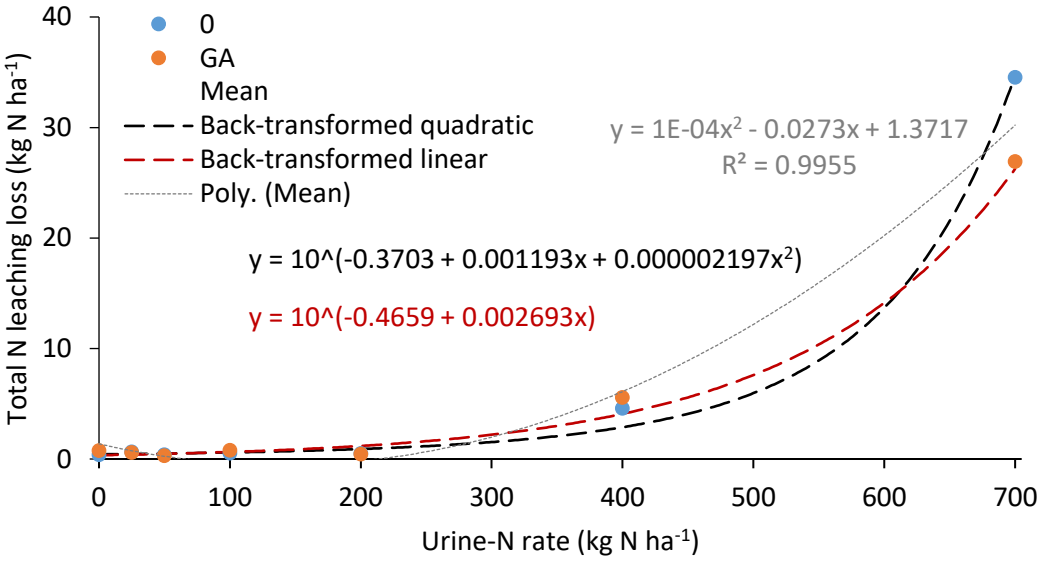


Figure 6.3 Relationship between urine-N rate and back-transformed total mineral nitrogen (NO_3^--N + NH_4^+-N) leaching loss (kg N ha^{-1}) at the end of the experimental period for lysimeters treated with or without application of gibberellic acid (8 g GA ha^{-1}) on 21 April 2016.

6.3.3 Herbage yield and nitrogen uptake

A significant linear and quadratic relationship was found between urine-N rate and cumulative herbage yield (t DM ha^{-1}) over the 6-month experimental period. There was a significant effect of GA ($P = 0.021$), however, there was no significant urine-N rate x GA interaction ($P = 0.712$). Herbage yield was overall higher when GA was applied ($P < 0.05$) (Figure 6.4). Across the different urine-N treatments, GA treated herbage was 0.2-1.4 t DM ha^{-1} (12-41%) higher than the control (no GA), except for the 50 kg N ha^{-1} urine treatment when it was 0.2 t DM ha^{-1} (8%) lower for the experimental period (Figure 6.4). Log-transformed means and LSDs are shown in Table 6.4.

Similarly, significant linear and quadratic relationships were found between urine-N rate and cumulative herbage N uptake (kg N ha^{-1}) over the 6-month experimental period. There was a significant effect of GA ($P = 0.039$), however, there was no significant urine-N rate x GA interaction ($P = 0.629$). Herbage N uptake was overall higher when GA was applied ($P < 0.05$) (Figure 6.5). Across the different urine-N treatments, GA treated herbage took up 3.6-31.9 kg N ha^{-1} (7-52%) more than the control (no GA), with the exception of the 50 kg N ha^{-1} urine treatment when it was 2.3 kg N ha^{-1} (5%) lower for the experimental period (Figure 6.5). Log-transformed means and LSDs are shown in Table 6.4.

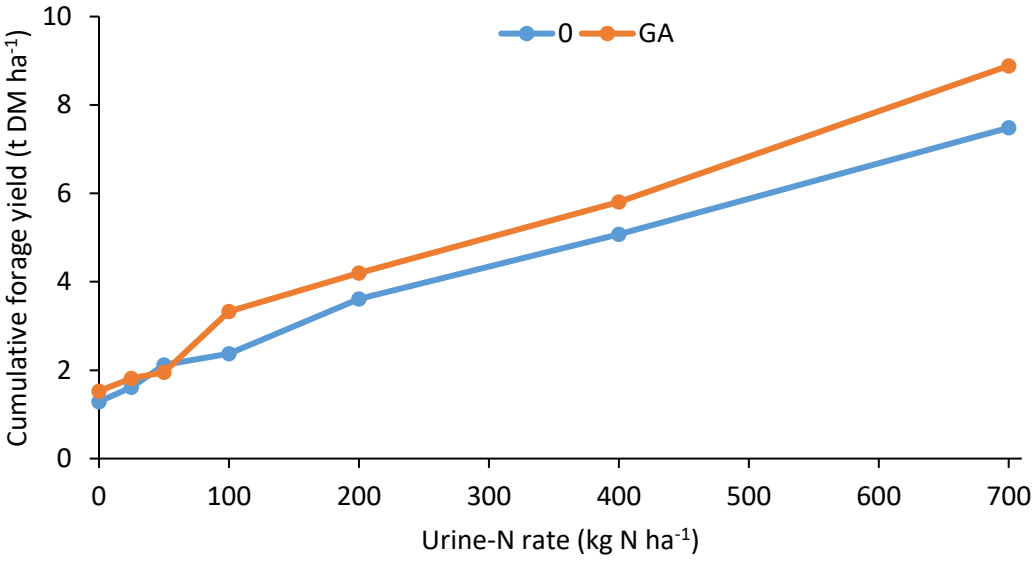


Figure 6.4 Relationship between urine-N rate and back-transformed total herbage dry matter yield (t DM ha^{-1}) at the end of the experimental period for lysimeters treated with or without application of gibberellic acid (8 g GA ha^{-1}) on 21 April 2016.

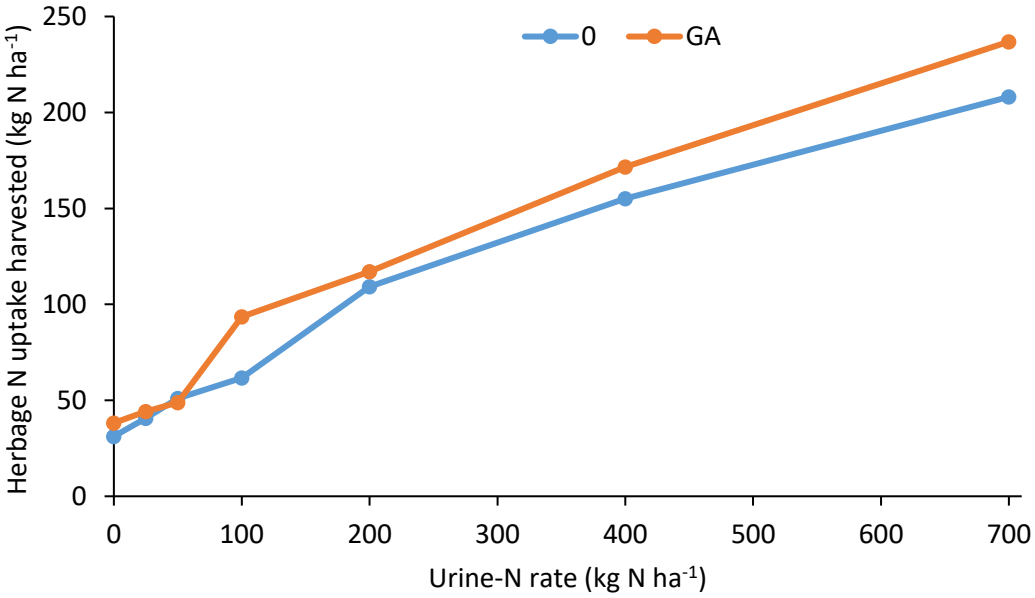


Figure 6.5 Relationship between urine-N rate and back-transformed herbage N uptake (kg N ha^{-1}) at the end of the experimental period for lysimeters treated with or without application of gibberellic acid (8 g GA ha^{-1}) on 21 April 2016.

Daily herbage N uptake (kg N ha^{-1} d^{-1}) was affected by GA application in the first two harvests (19 May 2016 and 2 August 2016) ($P < 0.01$), but not on the final harvest (6 October 2016) ($P = 0.715$) (Table 6.5). In the May and August harvests, daily N uptake was 17-24% higher for the GA treatment (1.13 and 0.24 kg N ha^{-1} d^{-1}, respectively), compared with the control (no GA) (1.12 and 0.19 kg N ha^{-1} d^{-1}, respectively) (Table 6.5). Across all three harvests, daily N uptake increased with increasing urine-N rate. However, there was no significant urine-N rate x GA interaction (Table 6.5).

Nitrogen content (%) of the herbage ranged from 1.7% to 5.4% across the three harvests during the experimental period (Table 6.6). The application of GA had no significant effect on the N content of the herbage (Table 6.6). Herbage N content increased significantly with increasing urine-N rate (Table 6.6). There was no urine-N rate x GA interaction (Table 6.6). The highest N contents were shown in the first harvest (19 May 2016) after treatment application, and tended to be lowest for the August harvest (Table 6.6).

Table 6.4 Log-transformed means and LSDs for N leaching loss, herbage yield, and N uptake for the experimental period (20 April 2016 to 17 October 2016). Lysimeters received urine applications ranging from 0 to 700 kg N ha^{-1} and were treated with or without application of gibberellic acid (8 g GA ha^{-1}) on 21 April 2016.

		Log N leaching loss (kg N ha^{-1})		Log herbage yield (t DM ha^{-1})		Log herbage N uptake (kg N ha^{-1})	
GA Treatment							
	0	0.096	a	0.451	b	1.878	b
	GA	0.122	a	0.513	a	1.938	a
	LSD (5%)	*0.221*		*0.052*		*0.056*	
	P-value	*0.685*		*0.021*		*0.039*	
Urine Treatment							
	0	-0.274	c	0.147	f	1.538	f
	25	-0.212	c	0.233	ef	1.627	ef
	50	-0.469	c	0.309	ef	1.698	e
	100	-0.193	c	0.449	d	1.881	d
	200	-0.332	c	0.590	c	2.054	c
	400	0.703	b	0.735	b	2.213	b
	700	1.484	a	0.911	a	2.346	a
	LSD (5%)	*0.413*		*0.097*		*0.105*	
	P-value	*<0.001*		*<0.001*		*<0.001*	
GA Treatment	**Urine Treatment**						
0	0	-0.425	c	0.110	i	1.494	g
0	25	-0.191	c	0.208	ghi	1.608	fg
0	50	-0.425	c	0.327	fg	1.708	ef
0	100	-0.286	c	0.375	f	1.790	e
0	200	-0.302	c	0.558	e	2.039	d
0	400	0.661	b	0.705	cd	2.191	bc
0	700	1.538	a	0.874	ab	2.318	ab
GA	0	-0.124	c	0.184	hi	1.582	fg
GA	25	-0.232	c	0.259	fgh	1.645	ef
GA	50	-0.514	c	0.291	fgh	1.688	ef
GA	100	-0.099	c	0.523	e	1.971	d
GA	200	-0.354	c	0.623	de	2.069	cd
GA	400	0.745	b	0.764	bc	2.234	ab
GA	700	1.430	a	0.949	a	2.375	a
	LSD (5%)	*0.585*		*0.138*		*0.149*	
P-value	GA x Urine	*0.942*		*0.712*		*0.629*	

Table 6.5 Log-transformed means and LSDs for daily N uptake at each harvest (DM yield x N% ÷ rotation length). Back-transformed data are in brackets. Lysimeters received urine applications ranging from 0 to 700 kg N ha^{-1} and were treated with or without application of gibberellic acid (8 g GA ha^{-1}) on 21 April 2016.

		Log daily herbage N uptake harvest 19 May 2016 (kg N ha^{-1} d^{-1})		Log daily herbage N uptake harvest 2 Aug 2016 (kg N ha^{-1} d^{-1})		Log daily herbage N uptake harvest 6 Oct 2016 (kg N ha^{-1} d^{-1})	
GA Treatment							
	0	0.051	(1.12)	-0.718	(0.19)	-0.431	(0.37)
	GA	0.119	(1.31)	-0.626	(0.24)	-0.414	(0.39)
	LSD (5%)	*0.049*		*0.061*		*0.098*	
	P-value	*0.008*		*0.004*		*0.715*	
Urine Treatment							
	0	-0.424	(0.38)	-1.116	(0.08)	-0.584	(0.26)
	25	-0.233	(0.59)	-0.989	(0.10)	-0.596	(0.25)
	50	-0.100	(0.79)	-0.974	(0.11)	-0.572	(0.27)
	100	0.105	(1.27)	-0.783	(0.16)	-0.459	(0.35)
	200	0.334	(2.16)	-0.593	(0.26)	-0.371	(0.43)
	400	0.489	(3.09)	-0.289	(0.51)	-0.321	(0.48)
	700	0.421	(2.64)	0.042	(1.10)	-0.052	(0.89)
	LSD (5%)	*0.092*		*0.115*		*0.183*	
	P-value	*<0.001*		*<0.001*		*<0.001*	
GA Treatment	**Urine Treatment**						
0	0	-0.465	(0.34)	-1.183	(0.07)	-0.613	(0.24)
0	25	-0.278	(0.53)	-1.005	(0.10)	-0.588	(0.26)
0	50	-0.094	(0.80)	-1.013	(0.10)	-0.524	(0.30)
0	100	0.039	(1.09)	-0.862	(0.14)	-0.556	(0.28)
0	200	0.319	(2.08)	-0.648	(0.22)	-0.360	(0.44)
0	400	0.468	(2.94)	-0.319	(0.48)	-0.331	(0.47)
0	700	0.368	(2.33)	0.007	(1.02)	-0.047	(0.90)
GA	0	-0.383	(0.41)	-1.049	(0.09)	-0.556	(0.28)
GA	25	-0.187	(0.65)	-0.973	(0.11)	-0.604	(0.25)
GA	50	-0.105	(0.79)	-0.935	(0.12)	-0.620	(0.24)
GA	100	0.172	(1.49)	-0.703	(0.20)	-0.362	(0.43)
GA	200	0.350	(2.24)	-0.539	(0.29)	-0.383	(0.41)
GA	400	0.510	(3.24)	-0.259	(0.55)	-0.312	(0.49)
GA	700	0.474	(2.98)	0.077	(1.19)	-0.058	(0.88)
	LSD (5%)	*0.130*		*0.162*		*0.259*	
P-value	GA x Urine	*0.739*		*0.929*		*0.806*	

Table 6.6 Mean herbage N content (%) and LSDs for each harvest. Lysimeters received urine applications ranging from 0 to 700 kg N ha^{-1} and were treated with or without application of gibberellic acid (8 g GA ha^{-1}) on 21 April 2016.

		Herbage N content harvest 19 May 2016 (%)	Herbage N content harvest 2 Aug 2016 (%)	Herbage N content harvest 6 Oct 2016 (%)
GA Treatment				
	0	3.8 ^{a}	1.9 ^{a}	2.3 ^{a}
	GA	3.8 ^{a}	1.9 ^{a}	2.2 ^{a}
	LSD (5%)	*0.2*	*0.1*	*0.2*
	P-value	*0.821*	*0.794*	*0.761*
Urine Treatment				
	0	2.8 ^{d}	1.8 ^{b}	2.6 ^{a}
	25	3.0 ^{d}	1.8 ^{b}	2.5 ^{a}
	50	3.0 ^{d}	1.7 ^{b}	2.3 ^{ab}
	100	3.5 ^{c}	1.9 ^{b}	2.4 ^{ab}
	200	4.2 ^{b}	1.8 ^{b}	2.3 ^{ab}
	400	5.1 ^{a}	1.9 ^{b}	2.0 ^{bc}
	700	5.3 ^{a}	2.6 ^{a}	1.8 ^{c}
	LSD (5%)	*0.3*	*0.3*	*0.4*
	P-value	*<0.001*	*<0.001*	*<0.001*
GA Treatment	**Urine Treatment**			
0	0	2.8 ^{e}	1.8 ^{b}	2.5 ^{ab}
0	25	2.9 ^{e}	1.8 ^{b}	2.6 ^{a}
0	50	3.0 ^{e}	1.7 ^{b}	2.2 ^{abc}
0	100	3.4 ^{cd}	1.8 ^{b}	2.2 ^{abc}
0	200	4.2 ^{b}	1.8 ^{b}	2.5 ^{ab}
0	400	5.2 ^{a}	1.9 ^{b}	2.0 ^{bcd}
0	700	5.4 ^{a}	2.7 ^{a}	1.8 ^{cd}
GA	0	2.9 ^{e}	1.9 ^{b}	2.6 ^{a}
GA	25	3.0 ^{e}	1.8 ^{b}	2.3 ^{abc}
GA	50	3.0 ^{de}	1.8 ^{b}	2.4 ^{ab}
GA	100	3.6 ^{c}	2.0 ^{b}	2.5 ^{ab}
GA	200	4.1 ^{b}	1.8 ^{b}	2.1 ^{abcd}
GA	400	5.0 ^{a}	1.8 ^{b}	2.0 ^{bcd}
GA	700	5.1 ^{a}	2.5 ^{a}	1.7 ^{d}
	LSD (5%)	*0.4*	*0.4*	*0.5*
P-value	GA x Urine	*0.848*	*0.617*	*0.495*

6.4 Discussion

6.4.1 Effect of GA on N leaching loss

The current experiment shows that GA has no effect on N leaching loss (Table 6.4) from perennial ryegrass-white clover treated with a range of rates of urine-N applications (0, 25, 50, 100, 200, 400, and 700 kg N ha^{-1}). This was consistent with the findings in Lysimeter Experiment 1 (Chapter 3) (Sections 3.3.2 and 3.4.3) where there was no effect of GA on N leaching loss from a 700 kg N ha^{-1} urine patch. In Lysimeter Experiment 1 (Chapter 3), not only did GA have no effect on N leaching loss but there was also no effect on DM yield or N uptake. This led to the hypothesis in Lysimeter Experiment 1 (Chapter 3) that the 700 kg N ha^{-1} urine patch was simply too high in N that any effect of the GA was swamped by the urine application. However, the findings of the current experiment where the effect of GA on N leaching loss from a wider range of urine-N rates was determined, has failed to reject the null hypothesis. To the author's knowledge, no other studies have measured the effect of an autumn GA application on N leaching loss. At this stage, the findings from both Lysimeter Experiment 1 (Chapter 3) and the current study indicate that an autumn application of GA is not suitable as a direct mitigation option for N leaching from grazed farm systems.

The N leaching losses from the 700 kg N ha^{-1} urine treatment in the current experiment were much lower than those observed in Lysimeter Experiment 1 (Chapter 3) (27-35 vs 186-224 kg N ha^{-1}, respectively). Total water inputs were much lower in the current experiment in the months of June and July (as a result of very high water inputs in May) compared with Lysimeter Experiment 1 (Chapter 3) which may have affected the amount of N leached. However, total water inputs for the first 6 months were higher for the current experiment (683 mm) than for Lysimeter Experiment 1 (Chapter 3) (596 mm). The difference in treatment application time is likely to have influenced the N leaching losses as timing of urine application has been shown to be important for N leaching losses (Selbie *et al.*, 2015). For the first 3 weeks following treatment application soil (10 cm) temperatures were much higher in the current experiment where application of urine occurred on 20 April 2016 (average 13.3°C; range 12-16°C), compared with those in Lysimeter Experiment 1 (Chapter 3) where urine was applied on 6 May 2014 (average 9.8°C; range 5-12°C). This appears to have allowed for increased herbage N uptake in the current experiment for the first harvest following urine application. Daily N uptake rates for the current experiment were much higher at 2.3-3 kg N ha^{-1} d^{-1}, compared with 1.6-1.7 kg N ha^{-1} d^{-1} for perennial ryegrass-white clover treated with 700 kg N ha^{-1}. If more N was taken up during this time, less would have been available to be leached.

6.4.2 Effect of urine-N rate on N leaching loss

The positive quadratic relationship between urine-N rate and N leaching loss shown in the current experiment (Figure 6.3) is consistent with other studies (Stout, 2003; Di & Cameron, 2007). However, neither of these other experiments had urine-N rates below 300 kg N ha^{-1} so the current experiment improves our knowledge of the relationship between urine-N rate and N leaching loss. Negative quadratic relationships have also been shown (Stout, 2003; Selbie, 2014), however, both of these previous experiments used some urine-N rates greater than the 700 kg N ha^{-1} used in the current experiment. Selbie (2014) measured total N in leachate rather than the total mineral N (NO_3^- + NH_4^+) measured in the current experiment which may also explain the slightly different relationship. The findings from the current experiment demonstrate that any mitigation option which reduces urine-N loading to levels below 400 kg N ha^{-1} is likely to significantly reduce leaching losses, compared with higher rates such as the 700 kg N ha^{-1} in the current experiment (Figure 6.3).

6.4.3 Effect of GA on forage DM yield

The overall 15% increase in DM yield over the experimental period for GA-treated perennial ryegrass-white clover (Table 6.4) was unexpected as it is in contrast to the findings in Lysimeter Experiment 1 (Chapter 3) (Section 3.3.3). Similarly, although not significant at the 5% level, DM yield of the 700 kg N ha^{-1} urine treatment was 1.4 t DM ha^{-1} (19%) higher when treated with GA, compared with the control (no GA) in the current experiment. In comparison, this was also not significant in Lysimeter Experiment 1 (Chapter 3) (Section 3.3.3), but the difference between the GA and non-GA herbage DM yield was much lower at 0.2 t DM ha^{-1} (0.9%). Other studies have also shown an increase in DM following the application of GA (Morgan & Mees, 1956, 1958; Finn & Nielsen, 1959; McGrath & Murphy, 1976; Matthew *et al.*, 2009; Jiang *et al.*, 2011; Ball *et al.*, 2012; Bryant, 2012; Parsons *et al.*, 2013; van Rossum *et al.*, 2013). Further increases in herbage DM yield have also been shown when GA was applied with fertiliser-N at rates of 20-80 kg N ha^{-1} (compared with the yield from the fertiliser-only treatments) (Morgan & Mees, 1958; Matthew *et al.*, 2009; Bryant, 2012; van Rossum *et al.*, 2013; Ghani *et al.*, 2014; Zaman *et al.*, 2014; Bryant *et al.*, 2016), however, none of these studies applied high rates of N (e.g. urine >200 kg N ha^{-1}) with GA. Herbage DM yields of the 700 kg N ha^{-1} urine treatment were much lower in the current experiment (7.5 and 8.9 t DM ha^{-1} for 0 and GA treatment, respectively) compared with Lysimeter Experiment 1 (Chapter 3) (24.3 and 24.5 t DM ha^{-1} for Urine and GA + Urine, respectively, (Section 3.3.3)). This is due to the shorter duration of the current experiment (6 months vs 17 months). However, the DM yields for the first 6 months of Lysimeter Experiment 1 (Chapter 3) (10.5-10.8 t DM ha^{-1}) were also higher than the current experiment. In Lysimeter Experiment 1 (Chapter 3), perennial ryegrass-white clover received a total of 80 kg N ha^{-1} fertiliser in the first 6 months (applied in April, August, and October) (Table 3.3, Section 3.2.4), whereas in the current experiment only 30 kg N ha^{-1} of fertiliser-N was applied (Section 6.2.4). This is likely to have influenced

the yield potential of the lysimeters in the current experiment and could help explain the lower herbage DM yields observed.

6.4.4 Effect of GA on forage N uptake and N content

The 15% overall increase in N uptake by the perennial ryegrass-white clover herbage when treated with GA is likely to relate to the increased herbage DM yield which was observed. This is due to GA having no effect on herbage N content (%). Nitrogen uptake (kg N ha^{-1}) for each harvest was calculated from herbage yield (kg DM ha^{-1}) x N%, these were summed to give the total N uptake for the experimental period. These findings are consistent with Zaman *et al.* (2014) and Parsons *et al.* (2013) who also showed an increased DM yield with no significant difference in N content, following GA application. Similarly, in other studies CP levels for GA-treated perennial ryegrass-white clover were either not significantly different (Matthew *et al.*, 2009), or increased (van Rossum *et al.*, 2013), compared to the control. In contrast to this, decreases in N% (McGrath & Murphy, 1976) and CP (Finn & Nielsen, 1959; Percival, 1980) have also been shown following GA application to perennial ryegrass, perennial ryegrass-white clover (Ghani *et al.*, 2014; Bryant *et al.*, 2016), and other forages (Morgan & Mees, 1958; Scurfield, 1958; Biddiscombe *et al.*, 1962). Increases in clover content (%) have also been observed with the application of GA to perennial ryegrass-white clover (van Rossum *et al.*, 2013; Bryant *et al.*, 2016). van Rossum *et al.* (2013) suggested that increase in CP following GA application to perennial ryegrass-white clover in their experiment appeared to be related to changes in clover content. An increase in clover content could increase the N% of the herbage. However, in a mixed sward with perennial ryegrass, the GA application could also decrease the N% of the ryegrass portion (McGrath & Murphy, 1976) which could potentially lead to no difference in N% of the sward overall. It is possible that this occurred in the current experiment, however, botanical composition was not measured.

6.4.5 Possible indirect effects of GA on N leaching loss

Despite autumn-applied GA having no direct effect on N leaching loss, the increased herbage dry matter yield and no difference in herbage N content shown in the current study indicate that an application of GA may allow for a reduction in N fertiliser application, as previously discussed in Section 3.4.3. This would reduce the N inputs to the farm system which reduces the amount of N cycling, and could therefore potentially reduce losses of N by leaching and denitrification (Whitehead & Edwards, 2015). Similarly, other studies have shown reductions in CP and N% following GA application (Morgan & Mees, 1958; Scurfield, 1958; Finn & Nielsen, 1959; Biddiscombe *et al.*, 1962; McGrath & Murphy, 1976; Percival, 1980; Ghani *et al.*, 2014; Bryant *et al.*, 2016), and although not observed in the current study, it is still possible that this may occur for different regrowth periods. If a reduction in herbage CP or N% does occur, this could lead to a reduction in urine-N excretion as discussed in Bryant *et al.* (2016),

but they also warn that an increase in clover % could negate any benefits from this on urine-N excretion. Ghani *et al.* (2014) modelled the effect that a reduction in herbage N% following GA application may have on urine-N excretion and N leaching. Reductions in N leaching were predicted to be as much as 29% in their model where GA was applied three times between April and August with a reduction in herbage N% from 3.9% to 3.2% during the April-August period and that this replaced a 30 kg N ha^{-1} fertiliser application at these times. In the most conservative scenario they modelled, GA was applied in April only with a reduction in herbage N concentration from 3.9% to 3.4% over the 1-month period following application, this predicted a 4% reduction in N leaching loss.

6.5 Conclusions

- An autumn application of gibberellic acid (8 g GA ha^{-1}) to perennial ryegrass-white clover had no effect on urine patch N leaching losses across a range of urine-N rates: 0, 25, 50, 100, 200, 400, and 700 kg N ha^{-1}.

- The GA application did however increase DM yields over the experimental period and therefore increased N uptake, although N content was unaffected. This indicates that the N uptake effect of GA was not substantial enough to effect total N leaching loss.

- Nitrogen leaching losses decreased with decreasing urine-N loading, particularly below a urine-N loading of 400 kg N ha^{-1}. Mitigation options should focus on reducing urine-N inputs to below 400 kg N ha^{-1} to reduce leaching losses from grazed systems.

Chapter 7

Conclusions and Recommendations

7.1 Conclusions

7.1.1 Effect of Italian ryegrass on N leaching losses

The following hypotheses were tested:

Hypothesis 1: That alternative forages such as Italian ryegrass and lucerne reduce N leaching compared with that of typical perennial ryegrass-white clover forage through mechanisms such as increased winter activity and root depth.

Hypothesis 3: That an increase in the uptake of urinary-N by plants reduces the amount of urinary-N leached.

Hypothesis 4: That Italian ryegrass decreases N leaching by inhibiting the first step of the nitrification process: ammonia oxidation.

Italian ryegrass was shown to decrease N leaching loss from an autumn urine patch, when compared with standard perennial ryegrass-white clover commonly found in New Zealand grazed systems. This was attributed to its ability to grow and take up more N during the cooler winter period which was consistent with Hypothesis #1 and Hypothesis #3. Evidence for this was shown in the ^{15}N balance where Italian ryegrass was more efficient than perennial ryegrass-white clover at taking up urine-N as shown by higher urine-^{15}N levels in the herbage of Italian ryegrass, than perennial ryegrass-white clover, and less urine-^{15}N was lost in the drainage of Italian ryegrass lysimeters (Chapter 3). In Chapter 4, Italian ryegrass was shown to have no effect on soil nitrifiers: ammonia-oxidising bacteria and archaea. Instead the effect of plants vs no plants had a greater effect. Thus, the findings of Chapter 4 confirm that the reduced N leaching loss from Italian ryegrass, when compared with perennial ryegrass-white clover relates to cool season uptake of urine-N. This finding reinforces that of others who have also measured lower leaching losses beneath Italian ryegrass than perennial ryegrass-white clover, and provides more evidence, through the use of the ^{15}N balance, that N uptake during the cool season (winter) was the primary mechanism involved. This study also found that there was no biological nitrification inhibition effect from Italian ryegrass, by showing no difference in ammonia-oxidising bacteria or archaea, or soil ammonium or nitrate levels between Italian ryegrass and perennial ryegrass. Therefore, Hypothesis #4 should be rejected.

7.1.2 Effect of an Italian ryegrass-plantain-white clover mixture on N leaching losses

The following hypotheses were tested:

Hypothesis 5: That an Italian ryegrass-plantain-white clover mixture would have a lower leaching loss than perennial ryegrass-white clover.

Hypothesis 6: That cows grazing the Italian ryegrass-plantain-white clover mixture have lower urine-N excretion, compared with perennial ryegrass-white clover.

Hypothesis 7: That the Italian ryegrass-plantain-white clover mixture would take up more N during the cool season than perennial ryegrass-white clover.

When Italian ryegrass was combined in a mixture with plantain and white clover in Chapter 5, large reductions in N leaching loss (45.5% - 88.9%) were measured, when compared to standard perennial ryegrass-white clover. This is consistent with Hypothesis #5. The two urine treatments accounted for the direct effect that the forages have on N leaching loss (Urine 700), as well as the influence that the forages have on urine-N excretion and subsequent N leaching loss (Urine Actual). These large reductions in leaching loss were again attributed to the Italian ryegrass-plantain-white clover mixture having higher winter growth and uptake of urine-N (consistent with Hypothesis #7). For the Urine Actual treatment, part of the 89% reduction in N leaching (compared with perennial ryegrass-white clover) was attributed to the lower concentration of urine-N collected from cows grazing the Italian ryegrass-plantain-white clover mixture (consistent with Hypothesis #6). The Italian ryegrass-plantain-white clover mixture was shown to be a promising forage type for grazed systems. This is because it can produce the same herbage dry matter yields as perennial ryegrass-white clover, has the ability to reduce urine-N excretion from grazing animals, and can significantly reduce N leaching losses, when compared to perennial ryegrass-white clover. To the author's knowledge, this is the first study to measure N leaching losses from an Italian ryegrass-plantain-white clover mixture, and to take into account the effect of this forage on urine-N excretion and subsequent N leaching losses.

7.1.3 Effect of lucerne on N leaching losses

The following hypothesis was tested:

Hypothesis 1: That alternative forages such as Italian ryegrass and lucerne reduce N leaching compared with that of typical perennial ryegrass-white clover forage through mechanisms such as increased winter activity and root depth.

Lucerne was shown to have much higher N leaching losses than perennial ryegrass-white clover (and Italian ryegrass) in Chapter 3. This was attributed to its poor herbage growth and thus minimal uptake of urine-N during the winter following urine application. To the author's knowledge, this study is the first to measure N leaching losses from urine deposited onto lucerne which is representative of grazed lucerne. Based on the high N leaching losses shown for lucerne in the current study, it may be advisable not to graze lucerne in late autumn (particularly in Canterbury), due to the potential for high N leaching losses to occur (as shown in Chapter 3). Instead farmers could cut and carry lucerne at this time of year, or feed out onto a feed pad to minimise deposition of urine onto lucerne fields prior to the winter drainage period. Therefore, in relation to lucerne, Hypothesis #1 should be rejected.

7.1.4 Effect of gibberellic acid on N leaching losses

The following hypotheses were tested:

Hypothesis 2: That gibberellic acid applied to forage in autumn increases both herbage growth and the uptake of urinary-N, subsequently reducing N leaching losses.

Hypothesis 8: That the application of GA to perennial ryegrass-white clover reduces N leaching from urine patches in autumn, but that there is a maximum urine-N rate above which this effect is negligible.

An application of GA in autumn was shown to have no effect on N leaching losses from urine in both Chapter 3 (perennial ryegrass-white clover, Italian ryegrass, and lucerne) and Chapter 6 (perennial ryegrass-white clover). In Chapter 3, GA also had no effect on total herbage dry matter yield and N uptake, or ^{15}N recovery when applied to a 700 kg N ha^{-1} urine patch. In Chapter 6, GA had no effect on N leaching across a range of urine-N rates: 0, 25, 50, 100, 200, 400, and 700 kg N ha^{-1}. However, overall herbage dry matter yields were increased by GA application in this experiment, this subsequently increased N uptake, however N content was unaffected by GA. This study was the first of its kind to measure the effect of GA application on N leaching from grazed systems. At this stage an autumn application of GA (8 g GA ha^{-1}) was shown to have no direct effect on urine patch N leaching losses and so is not recommended as a direct mitigation tool for N leaching losses in grazed systems. Therefore, Hypothesis #2 (relating to N leaching losses) and Hypothesis #8 should be rejected.

7.1.5 Effect of urine-N rate on N leaching loss

The results shown in Chapter 5 and 6 confirm that lower urine-N loading rates result in lower N leaching losses. Significantly lower N leaching losses were shown in Chapter 5 where urine-N rate was 508 kg N ha^{-1} (Italian ryegrass-plantain-white clover mixture), when compared with 664 kg N ha^{-1} (perennial ryegrass-white clover). Similarly, in Chapter 6, N leaching losses were measured for a range of urine-N rates: 0, 25, 50, 100, 200, 400, and 700 kg N ha^{-1} and were shown to decrease with decreasing urine-N

rate, particularly below 400 kg N ha^{-1}. Thus, based on these findings, it is recommended that mitigation options should aim to reduce urine-N excretion from grazing animals to deposition rates lower than 400 kg N ha^{-1} in order to reduce N leaching losses from grazed agricultural systems. Others have also shown decreased N leaching with decreasing urine-N application rates (e.g. Stout, 2003; Di & Cameron, 2007; Selbie, 2014). However, the current study improves our knowledge of the relationship between urine-N rate and N leaching loss by including a number of low urine-N rates (25, 50, 100, and 200 kg N ha^{-1}).

7.1.6 Seasonal/climate effect on N leaching loss

A perennial ryegrass-white clover forage with 700 kg N ha^{-1} urine applied was included for the experiments in Chapter 3, Chapter 5, and Chapter 6. This provides a direct comparison of N leaching losses for three different urine application years: urine was applied in autumn of 2014 (May) (Chapter 3), 2015 (March) (Chapter 5), and 2016 (April) (Chapter 6). Nitrogen leaching losses were 186, 113, 35 kg N ha^{-1}, for each of these urine applications, respectively. This reinforces the large variation in N leaching losses which can occur between years (likely due to differences in weather, climate, growing conditions for forage, and timing of autumn urine application). Thus it is important to use long-term data, rather than measurements for individual years, to determine farm N leaching losses for regulatory purposes.

7.2 Recommendations for further research

Some of the key research questions arising from the current study are:

1. What is the best way to manage these alternative forages: Italian ryegrass and Italian ryegrass-plantain-white clover mixture for use in grazed agricultural systems? How can they be incorporated into current grazed systems?

2. What proportion of a farm would need to be planted with these proposed alternative forages (Italian ryegrass, and Italian ryegrass-plantain-white clover mixture) in order to significantly reduce whole farm N leaching losses? A modelling study by Khaembah *et al.* (2014) indicated that a New Zealand farm planted with 20% diverse pasture (containing a mixture of perennial ryegrass-white clover, chicory (*Chicorium intybus*), plantain, prairie grass (*Bromus wildenowii*), and either lucerne (*Medicago sativa*) or red clover (*Trifolium pratense*)) could reduce whole farm N excretion by ~3%, and for a farm planted with 50% diverse pasture this was a 5-8% reduction.

3. What percentage of plantain is required in the Italian ryegrass-plantain-white clover mixture to still influence urine-N excretion and reduce N leaching losses? (current study was 42% plantain).

4. What is the mechanism behind the reduced urine-N excretion from animals grazing forages containing plantain?

5. Does plantain itself inhibit nitrification in grazed forage systems? Can some of the reduction in N leaching from Italian ryegrass-plantain-white clover mixtures be attributed this? If nitrification inhibition is shown, does aucubin, a plant secondary metabolite found in plantain, play a role in nitrification inhibition?

6. How will N leaching losses under these alternative forages vary across different regions and soil types, compared to perennial ryegrass-white clover? Are other alternative forages more suitable in other regions around New Zealand?

7. Does lucerne dry the soil out over the summer period so that it takes longer to wet up in winter, compared to perennial ryegrass-white clover? If deeper (>0.7 m) lysimeters were used, would N leaching losses from lucerne (which received autumn urine application) still be significantly higher than those from perennial ryegrass-white clover? Can lucerne extract urine-N from depth (below 0.7 m) during spring-summer when its rapid growth occurs, resulting in subsequently less N leaching? A deep ^{15}N injection field plot experiment could be carried out to determine this (e.g. Huang *et al.*, 1996; Malcolm *et al.*, 2015).

8. Are N leaching losses from lucerne lower if urine is applied earlier in the autumn? To minimise N leaching losses, what time of year is best to take grazing animals off lucerne and feed them by cut and carry?

7.3 Implications

This research has revealed some potential tools which farmers could use to reduce their N leaching losses into the future:

- By optimizing forage growth and N uptake, N leaching loss can be reduced using:
  - Forages which are more winter-active e.g. Italian ryegrass
  - Forages which reduce urine-N excretion e.g. Italian ryegrass-plantain-white clover mix

- GA and grazed lucerne are not recommended as mitigation tools at this stage

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Appendix A

Soil map

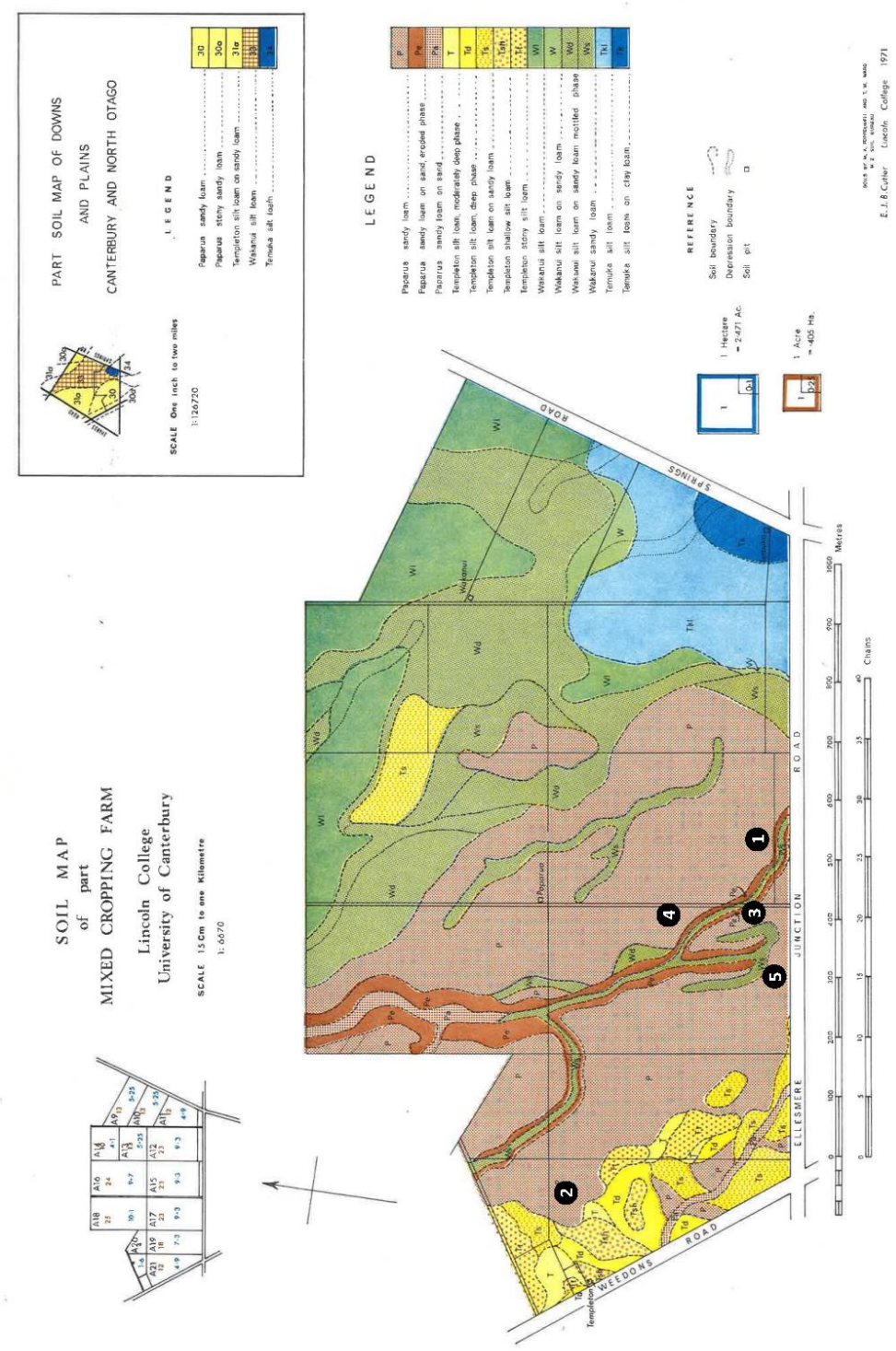

Figure A 1 Soil map of the Lincoln University Research Dairy Farm showing lysimeter collection sites: 1 = Chapter 3 RGWC and Italian RG lysimeters; 2 = Chapter 3 Lucerne lysimeters; 3 = Chapter 5 Italian-Plantain Mix lysimeters and Chapter 4 soil collection site; 4 = Chapter 5 RGWC lysimeters; 5 = Chapter 6 RGWC lysimeters.

Appendix B

Rainfall and irrigation simulation system

The rainfall and irrigation simulation system was used in Lysimeter Experiment 1 (Chapter 3) and Lysimeter Experiment 2 (Chapter 5). In Gibberellic Acid Experiment (Chapter 6) this system was used to schedule manual applications of irrigation and simulated rainfall. This system has previously been described by Malcolm (2013, Appendix D). This system was set up to best simulate actual rainfall and irrigation events, in relation to application intensity, rate and frequency. It also aims to generate sufficient drainage water during winter and spring to achieve a complete nitrogen breakthrough curve (although this was not achieved for Lysimeter Experiment 1 or Lysimeter Experiment 2 in the current study). A description of the programme below is as outlined in Malcolm (2013).

Programme description

The system operates on predefined daily climate parameters derived from data collected by the NIWA Broadfield weather station, Canterbury, New Zealand. Daily average rainfall and evapotranspiration data to the 75th percentile between 1975 and 1998/99 are used as a basis for climate prediction, and around which the programme operates. The system was calibrated to apply water at a rate of 1000 mL per minute in 0.5 mm bursts (Carrick *et al.*, 2010).

Key definitions:

Climate value: The daily seasonal requirement of water based upon previous climate data to the 75th percentile (rainfall and evapotranspiration) and leachate generation (Figure B 1; yellow line).

Climate accumulation line: The main reference line that is derived from accumulating daily *climate values*.

Target line: A line that randomly tracks within plus or minus 20 mm of the *climate accumulation* line to create variability and randomness around a constant reference. Note: this only occurs under rainfall simulation mode.

Tally: Accumulation of rainfall, simulated rain and irrigation.

Application: Amount of water to be applied by the system (mm).

Rainfall simulation mode (April to September)

At midnight each day, the programme determines new *climate accumulation* and *target* values. The new *climate accumulation* value is calculated by the addition of the previous day's *climate accumulation* value and the current *climate value* shown in Figure B 1. The new *target* is the addition of the *climate value* and previous *target*.

When the *tally* is less than or equal to the *target*, a new *target* is created by a random number generator, within the defined boundaries of the *climate line*. This new *target* may be below or above the *tally*. If the new *target* is less than the *tally*, no water is applied, and the system repeats the first operation each day until the condition of positive *application* is created. If the new *target* is greater than the *tally* then the difference between the *target* and *tally* will be applied as simulated rain.

Examples of "rainfall simulation mode" are shown in Figure B 3, Figure B 5, Figure B 7, and Figure B 9. These illustrate the random nature of the *target* line around the *climate accumulation line*, and the event of simulated rain *application*. The concept of a randomly fluctuating *target* is to bring variability into the system which is a characteristic of an actual climate, and therefore no one season is replicated in the exact same way. The black bars on the graph indicate randomly generated *application* amounts.

The *application* of simulated rain is done so by a randomly generated 'pulse pattern,' otherwise known as 'random intensity.' The rate of intensity (mm hr^{-1}) is weighted towards lower values, and the overall range of these possible values is weighted by the actual amount to be applied. Lower *application* amounts equal lower intensity range rates; higher *application* amounts equal higher possible range rates.

Irrigation mode (October to March)

The procedure for irrigation *application* is similar to that of the rainfall simulation methodology, however, the amount, frequency and intensity is defined by user-set variables. It uses the *climate accumulation line* as a reference point instead of the fluctuating *target* line and therefore *application* trends are more linear. This is set to simulate irrigation through a centre pivot.

In these experiments, the irrigation regime was comprised of applications approximately every three days, with single applications of 12 mm at an intensity of 20 mm h^{-1} in October, 15 mm (20 mm h^{-1} intensity) from November to January, then 18 mm (20 mm h^{-1} intensity) from mid-January to March for Lysimeter Experiment 1 (Chapter 3), and 12 mm (20 mm h^{-1} intensity) from Feburary to April for Lysimeter Experiment 2 (Chapter 5). In the event of rain, the time interval between applications was extended in order to remain on track with the *climate accumulation* line.

Examples of “irrigation mode” are shown in Figure B 4 and Figure B 8. These illustrate actual data from the 2014/2015 and 2015/2016 irrigation seasons (respectively) when the system was in ‘irrigation mode.’ Unlike for the previous examples of “rainfall simulation mode” where there was variability around the *target* amount and frequency, Figure B 4 and Figure B 8 both illustrate the consistent pattern of *applications* when in ‘irrigation mode,’ which are typical of irrigation practice, and more specifically that of a centre pivot. Also note that the *target* line (dotted red line) tracks the exact same path of the *climate accumulation line* (yellow line).

Exceptions

All *applications* halt for real rainfall events and defined environmental conditions (e.g. wind speed greater than 3 m sec^{-1}). For rainfall simulation, the amount of real rainfall is deducted from the quantified *application* amount and added to the *tally*.

Further, the daily climate values (Figure B 1) are subject to real climate conditions, and can be adjusted for either wet or dry years to achieve complete breakthrough curves.”

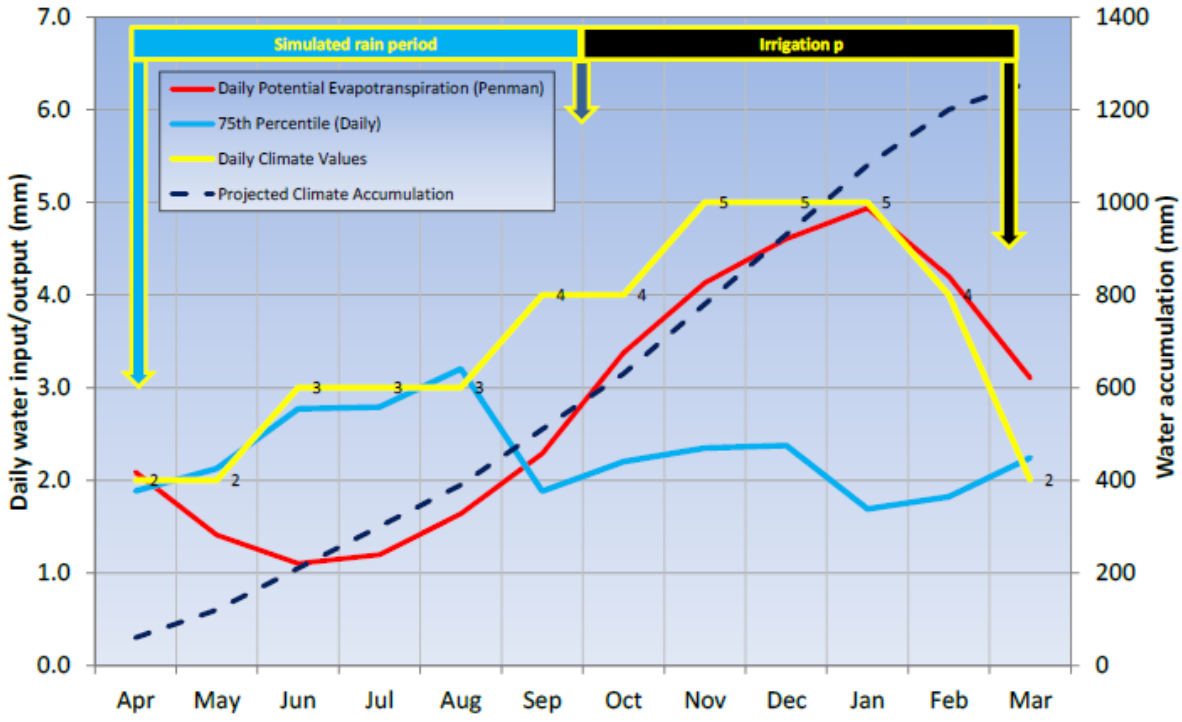

Figure B 1 The model controlling the automatic simulated rainfall and irrigation programme.

B.1 Lysimeter Experiment 1 (Chapter 3) detailed rainfall and irrigation

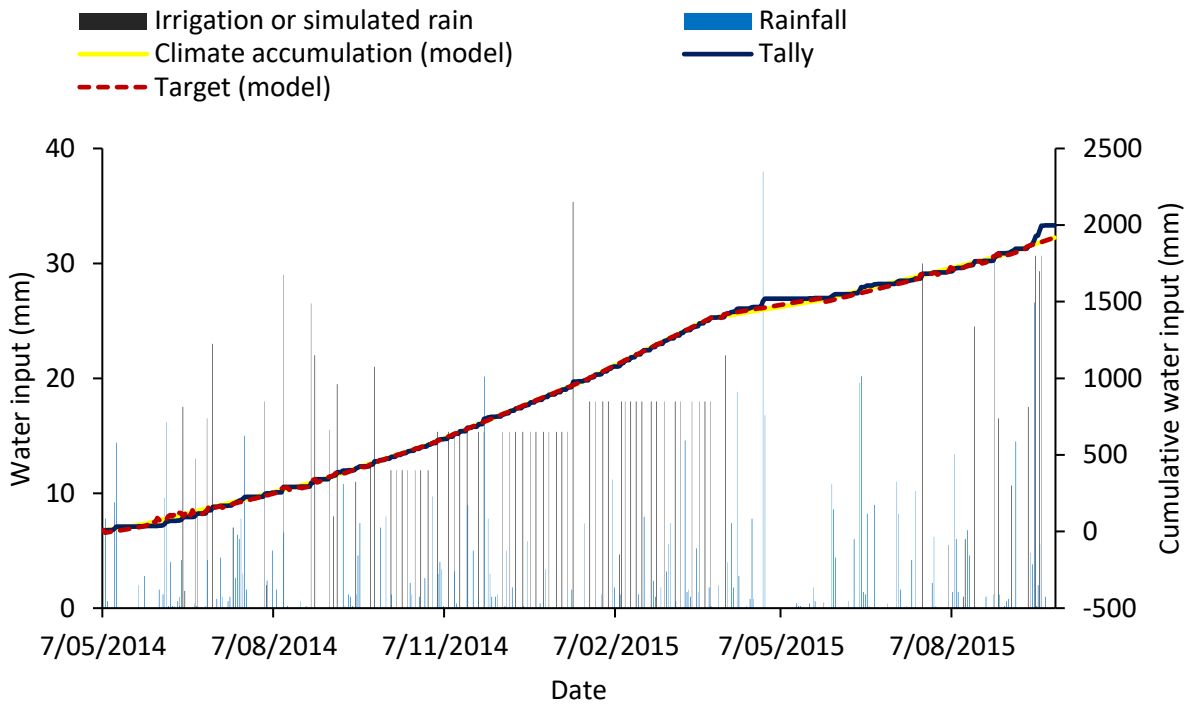

Figure B 2 Rainfall, simulated rain and irrigation, tally, climate accumulation and target of Lysimeter Experiment 1 (Chapter 3) throughout the experimental period between May 2014 and October 2015.

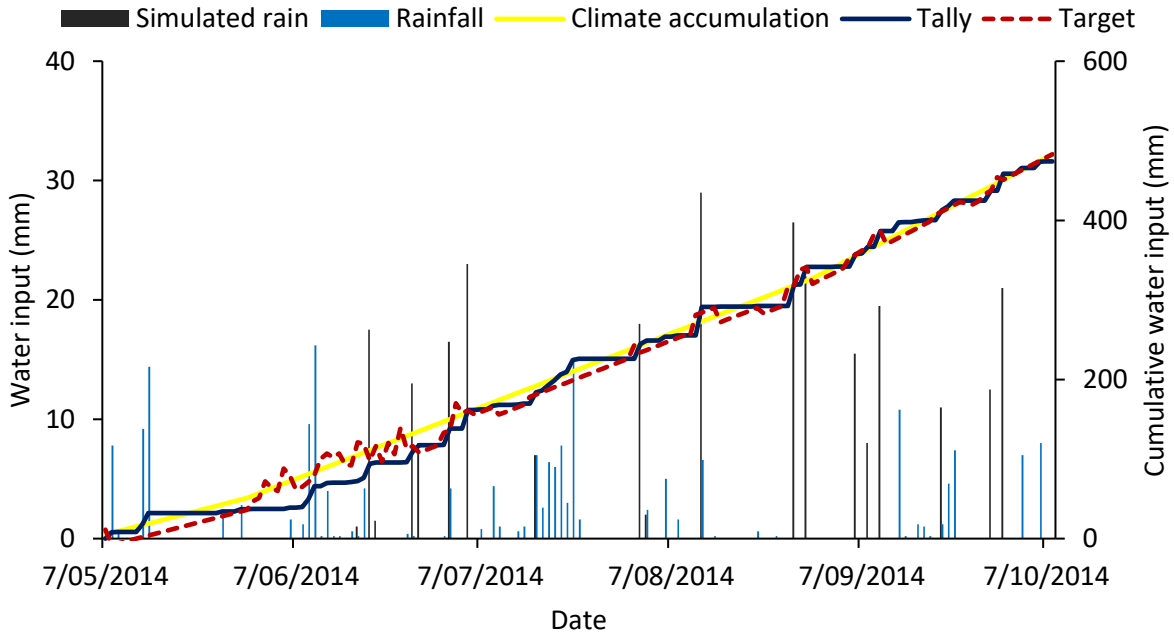

Figure B 3 Rainfall, simulated rain, climate accumulation, tally and target of Lysimeter Experiment 1 (Chapter 3) under 'rain simulation mode' between May 2014 and October 2014.

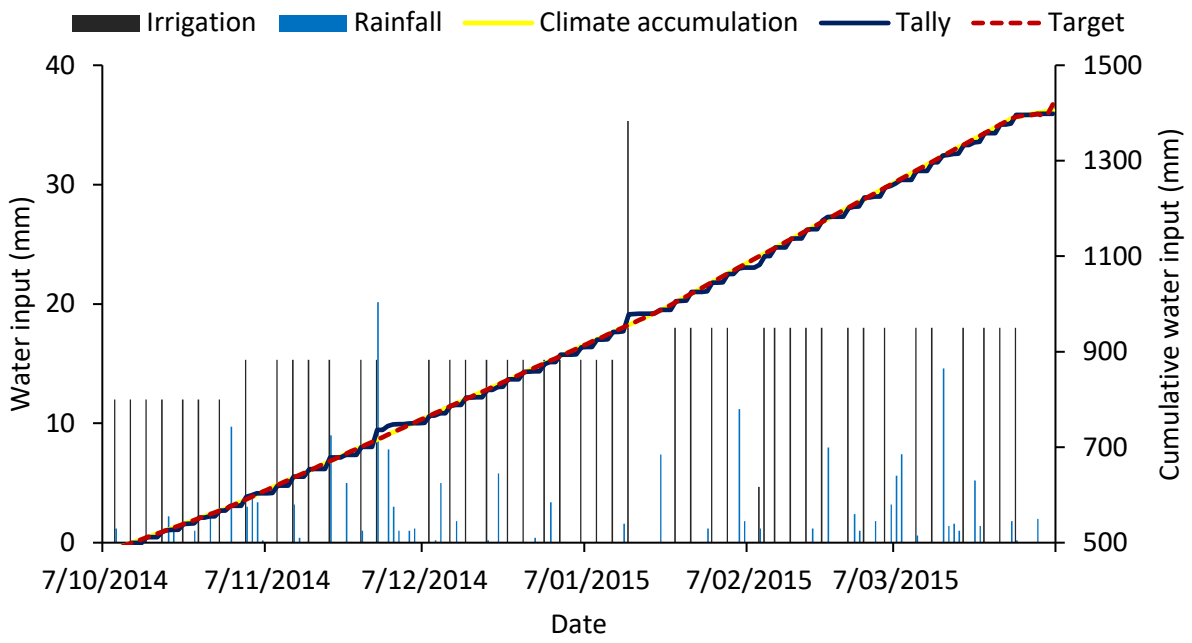

Figure B 4 Rainfall, irrigation, climate accumulation, tally and target of Lysimeter Experiment 1 (Chapter 3) under 'irrigation mode' between October 2014 and April 2015.

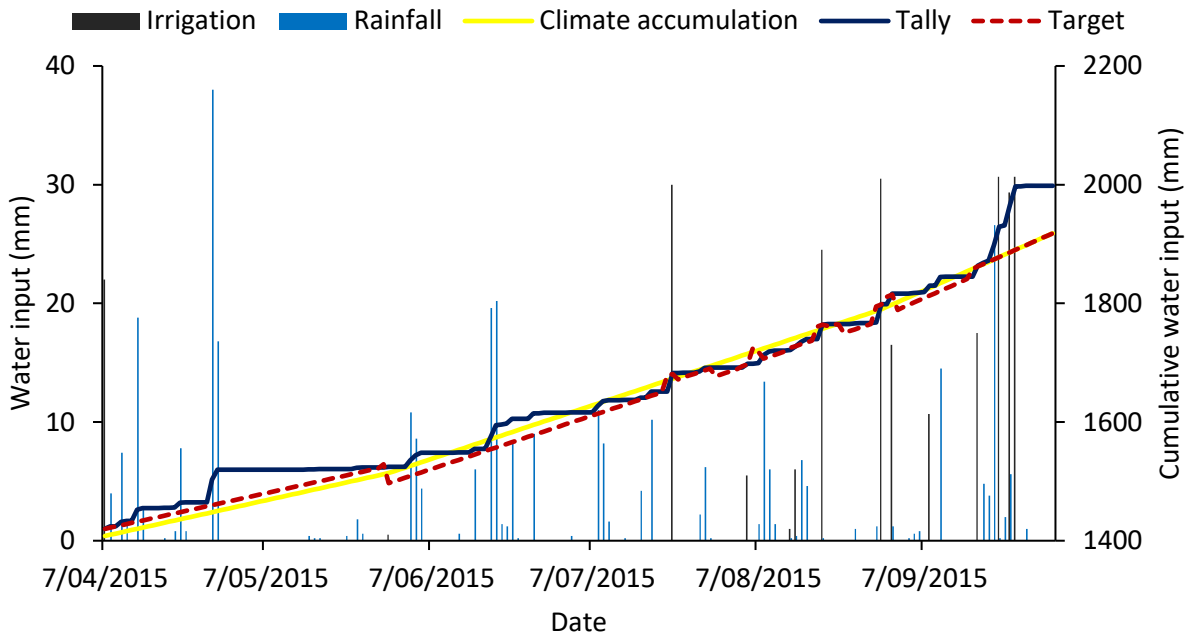

Figure B 5 Rainfall, simulated rain, climate accumulation, tally and target of Lysimeter Experiment 1 (Chapter 3) under 'rain simulation mode' between April 2015 and October 2015.

B.2 Lysimeter Experiment 2 (Chapter 5) detailed rainfall and irrigation

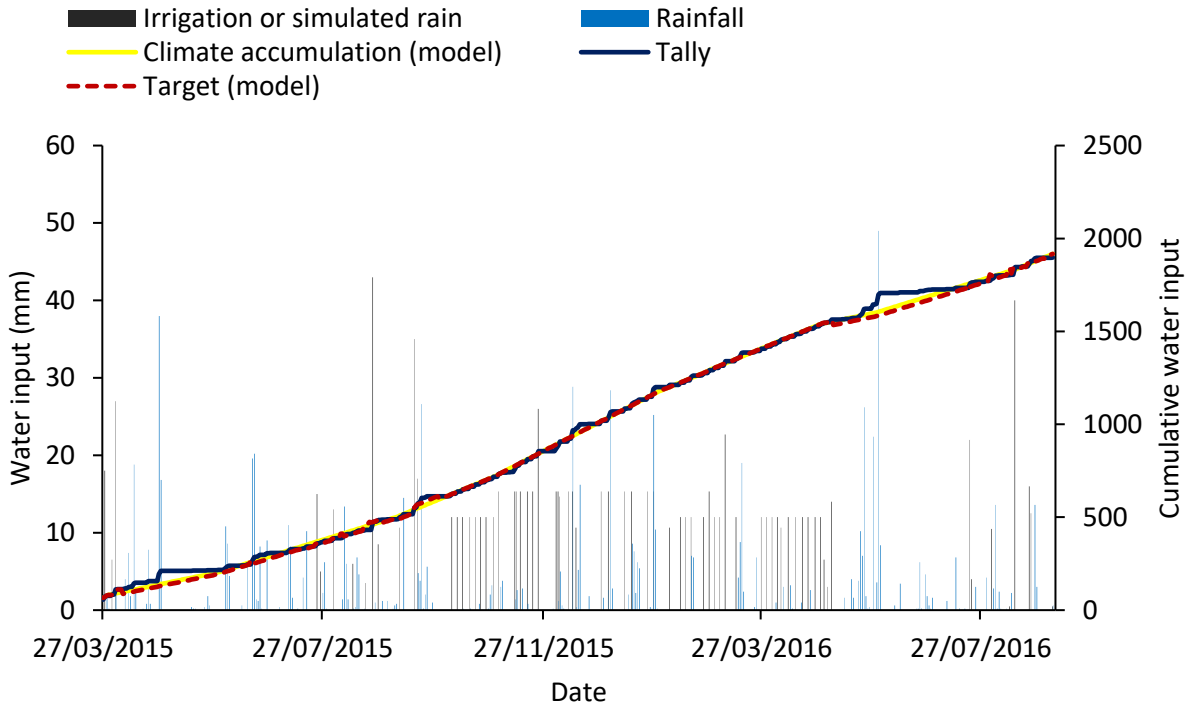

Figure B 6 Rainfall, simulated rain and irrigation, tally, climate accumulation and target of Lysimeter Experiment 2 (Chapter 5) throughout the experimental period between March 2015 and September 2016.

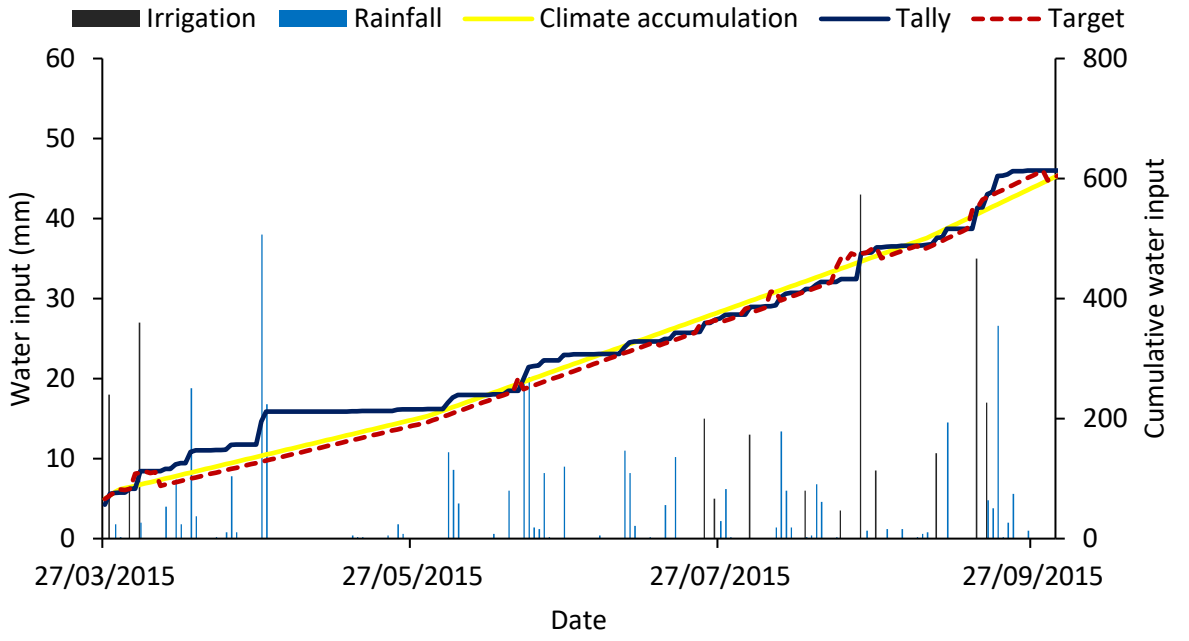

Figure B 7 Rainfall, simulated rain, climate accumulation, tally and target of Lysimeter Experiment 2 (Chapter 5) under 'rain simulation mode' between March 2015 and October 2015.

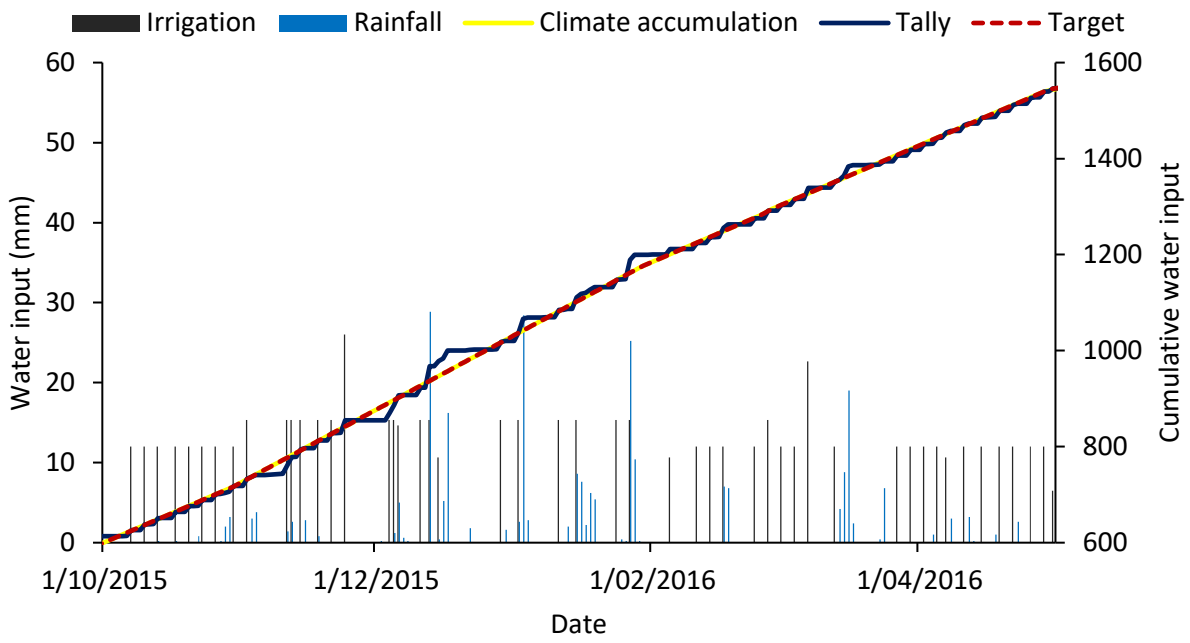

Figure B 8 Rainfall, irrigation, climate accumulation, tally and target of Lysimeter Experiment 2 (Chapter 5) under 'irrigation mode' between October 2015 and April 2016.

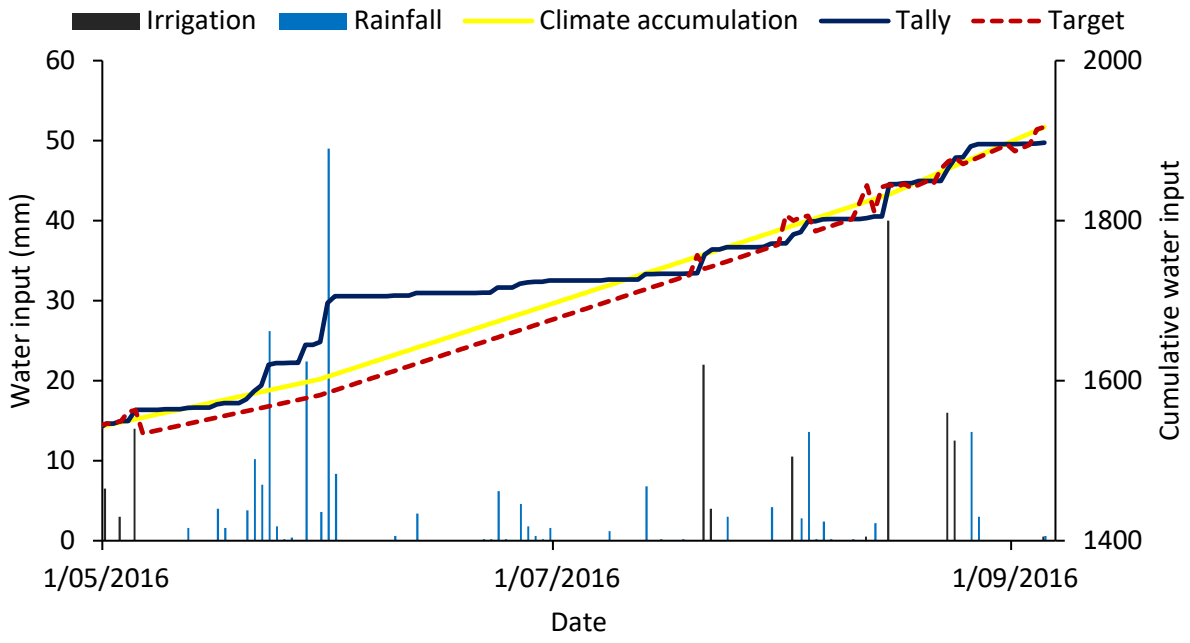

Figure B 9 Rainfall, simulated rain, climate accumulation, tally and target of Lysimeter Experiment 2 (Chapter 5) under 'rain simulation mode' between May 2016 and September 2016.

Appendix C

Herbage data

C.1 Herbage data for Lysimeter Experiment 1 (Chapter 3)

Table C 1 Results of herbage testing for the 5 December 2014 harvest through a commercial laboratory (Analytical Research Laboratories, NZ).

Plot #	Forage type	Treatment	Total N % w/w	P % w/w	K % w/w	S % w/w	Ca % w/w	Mg % w/w	Na % w/w	Fe mg kg^{-1}	Mn mg kg^{-1}	Cu mg kg^{-1}	Zn mg kg^{-1}	B mg kg^{-1}
1	Italian	Urine	2.29	0.35	2.92	0.27	0.33	0.11	0.15	58	79	3.6	28	6.1
2	Italian	Control	2.26	0.44	2.76	0.29	0.38	0.14	0.15	60	65	4.1	23	6.7
3	Italian	GA + Urine	2.39	0.36	3.27	0.30	0.38	0.18	0.14	69	76	3.4	22	6.0
4	Lucerne	Control	3.13	0.22	1.33	0.25	2.01	0.23	0.13	41	36	3.3	23	46
5	Lucerne	GA + Urine	2.13	0.19	2.20	0.21	0.99	0.11	0.04	34	25	2.3	16	36
6	Lucerne	Urine	2.48	0.20	2.80	0.26	0.83	0.11	0.04	36	33	2.8	23	37
7	RGWC	GA + Urine	3.21	0.37	3.37	0.31	0.56	0.15	0.39	86	100	5.0	30	13
8	RGWC	Control	3.67	0.42	2.98	0.31	0.74	0.19	0.43	93	78	5.5	34	17
9	RGWC	Urine	2.53	0.38	2.90	0.29	0.41	0.14	0.29	67	72	4.0	22	5.2
10	Italian	Urine	2.14	0.35	2.89	0.26	0.40	0.12	0.20	61	77	3.6	30	6.1
11	Italian	Control	2.06	0.38	2.52	0.26	0.33	0.14	0.17	63	96	3.7	21	6.0
12	Italian	GA + Urine	2.13	0.34	2.88	0.26	0.36	0.14	0.15	59	62	3.4	21	5.2
13	RGWC	Control	4.42	0.42	3.02	0.31	1.23	0.21	0.39	120	60	5.7	43	22
14	RGWC	GA + Urine	2.98	0.39	3.12	0.32	0.50	0.14	0.26	88	100	4.6	28	11
15	RGWC	Urine	3.36	0.37	3.04	0.31	0.53	0.18	0.40	110	73	4.3	25	8.4
16	Lucerne	GA + Urine	2.98	0.21	2.64	0.26	1.24	0.13	0.05	45	34	2.8	18	39
17	Lucerne	Control	3.62	0.25	1.09	0.28	1.93	0.24	0.24	66	38	3.3	20	40
18	Lucerne	Urine	2.73	0.19	2.53	0.24	1.24	0.13	0.06	39	35	3.0	19	37
19	RGWC	Urine	2.98	0.33	3.26	0.30	0.48	0.16	0.30	80	83	3.8	30	7.5
20	RGWC	GA + Urine	3.44	0.35	2.88	0.31	0.52	0.16	0.75	68	85	4.0	30	9.3
21	RGWC	Control	4.20	0.37	1.98	0.29	0.97	0.20	0.64	78	56	4.9	32	19
22	Lucerne	Urine	2.40	0.21	2.78	0.28	1.12	0.13	0.05	42	39	2.7	19	42
23	Lucerne	GA + Urine	2.94	0.21	2.54	0.25	1.26	0.13	0.06	37	29	2.8	20	35

Table C 2 Results of herbage testing for the 5 December 2014 harvest through a commercial laboratory (Analytical Research Laboratories, NZ) <continued>

Plot #	Forage type	Treatment	Total N % w/w	P % w/w	K % w/w	S % w/w	Ca % w/w	Mg % w/w	Na % w/w	Fe mg/kg	Mn mg/kg	Cu mg/kg	Zn mg/kg	B mg/kg
24	Lucerne	Control	2.70	0.24	1.42	0.27	1.67	0.24	0.16	41	22	2.5	20	34
25	Italian	GA + Urine	2.11	0.36	2.68	0.26	0.39	0.12	0.23	50	62	3.3	21	5.6
26	Italian	Control	2.28	0.41	2.55	0.32	0.41	0.15	0.23	61	70	4.0	22	8.4
27	Italian	Urine	2.68	0.34	2.94	0.29	0.42	0.16	0.22	59	56	3.1	26	5.6
28	RGWC	Control	3.71	0.40	2.43	0.31	0.77	0.19	0.53	77	67	5.4	30	16
29	RGWC	GA + Urine	3.74	0.34	2.80	0.30	0.57	0.18	0.85	70	79	4.5	32	9.4
30	RGWC	Urine	3.25	0.35	2.86	0.34	0.57	0.21	0.48	70	85	4.1	24	8.2
31	Italian	Control	2.20	0.45	2.85	0.33	0.38	0.15	0.13	67	100	4.2	28	6.8
32	Italian	Urine	2.67	0.33	2.89	0.31	0.40	0.21	0.30	82	86	3.7	30	6.0
33	Italian	GA + Urine	2.21	0.34	2.58	0.24	0.36	0.11	0.20	51	77	3.3	30	5.3
34	Lucerne	GA + Urine	3.01	0.19	2.46	0.26	1.20	0.12	0.05	38	33	3.1	23	33
35	Lucerne	Control	2.77	0.19	1.56	0.22	1.28	0.15	0.10	36	24	2.6	20	39
36	Lucerne	Urine	2.92	0.20	2.44	0.26	1.31	0.14	0.06	41	27	2.5	20	37
37	RGWC	Control	3.99	0.39	3.07	0.34	0.75	0.18	0.36	98	75	5.3	38	18
38	RGWC	GA + Urine	2.96	0.34	2.94	0.28	0.51	0.14	0.46	73	82	3.7	33	9.5
39	RGWC	Urine	2.52	0.35	2.92	0.28	0.48	0.14	0.30	79	79	3.8	27	7.3
40	Lucerne	Urine	2.84	0.23	2.64	0.27	1.20	0.12	0.05	47	37	3.1	25	39
41	Lucerne	GA + Urine	2.15	0.20	2.18	0.24	1.11	0.12	0.05	34	26	3.1	19	33
42	Lucerne	Control	3.03	0.23	1.29	0.27	1.75	0.21	0.14	44	30	2.5	22	39
43	Italian	GA + Urine	2.72	0.29	2.89	0.29	0.39	0.20	0.23	62	86	3.2	31	6.2
44	Italian	Urine	3.53	0.28	2.78	0.34	0.47	0.19	0.38	65	98	4.2	31	6.5
45	Italian	Control	1.92	0.39	2.78	0.31	0.33	0.14	0.12	53	72	3.2	20	5.9
Fresh mixed pasture		***Optimum range***	***3-4***	***0.35-0.45***	***2.5-3***	***0.27-0.32***	***0.25-0.5***	***0.18-0.22***	***0.1-0.25***	***50-60***	***25-30***	***6-8***	***14-20***	***-***
Lucerne		***Optimum range***	***4.5-5***	***0.26-0.7***	***2.5-3.8***	***0.26-0.5***	***0.51-3***	***0.31-1***	***0.02-0.05***	***30-250***	***30-100***	***11-30***	***21-70***	***30-80***

Table C 3 Lysimeter Experiment 1 (Chapter 3) forage quality data per harvest (as analysed by NIRS).

	Days since treatment application		-2	47	92	131	156	182	201	224	250	280	307	338	378	502
	Forage	Treatment	5 May 14	23 Jun 14	7 Aug 14	15 Sep 14	10 Oct 14	5 Nov 14	24 Nov 14	17 Dec 14	12 Jan 15	11 Feb 15	10 Mar 15	10 Apr 15	20 May 15	21 Sep 15
WSC mg g^{-1} DM	RGWC	Control	76	149	128	151	119	211	303	354	281	178	149	125	150	265
	RGWC	Urine	58	99	83	136	115	224	407	531	298	171	145	113	131	225
	RGWC	GA + Urine	75	112	96	152	106	201	370	504	319	177	148	131	147	247
	Italian RG	Control	86	272	238	268	232	375	509	597	377	184	173	172	162	271
	Italian RG	Urine	107	113	82	155	111	301	507	608	403	191	169	163	154	255
	Italian RG	GA + Urine	120	106	93	163	131	336	552	632	438	206	185	187	171	271
	Lucerne	Control	63	99	-	75	81	-	255	-	224	121	106	-	95	119
	Lucerne	Urine	64	89	-	76	82	-	270	-	211	124	97	-	81	117
	Lucerne	GA + Urine	69	81	-	69	85	-	280	-	223	121	94	-	95	107
CP mg g^{-1} DM	RGWC	Control	266	242	261	260	236	188	254	225	188	188	223	221	231	196
	RGWC	Urine	255	305	280	249	225	171	186	151	171	186	227	229	237	202
	RGWC	GA + Urine	251	286	283	257	242	188	208	167	169	178	211	210	227	199
	Italian RG	Control	277	180	162	170	141	107	138	114	116	126	157	156	191	145
	Italian RG	Urine	279	324	275	240	243	154	170	107	98	120	150	160	188	148
	Italian RG	GA + Urine	264	318	278	232	230	136	146	104	94	110	147	153	186	145
	Lucerne	Control	222	300	-	319	284	-	191	-	212	216	247	-	243	292
	Lucerne	Urine	225	332	-	316	269	-	171	-	198	195	233	-	235	284
	Lucerne	GA + Urine	232	331	-	319	276	-	163	-	195	188	234	-	232	298
ME MJ kg^{-1} DM	RGWC	Control	11.4	12.1	12.1	12.0	11.4	11.8	11.9	11.8	11.2	11.9	12.0	11.8	12.1	12.6
	RGWC	Urine	11.4	12.0	11.8	11.8	11.4	11.9	11.8	12.0	11.1	11.7	11.9	11.6	12.0	12.4
	RGWC	GA + Urine	11.4	11.9	11.9	11.9	11.4	11.8	11.7	11.9	11.2	11.8	11.9	11.7	12.0	12.6
	Italian RG	Control	11.1	12.7	12.4	12.5	11.9	12.4	11.5	11.4	9.9	10.4	11.3	11.6	12.0	12.2
	Italian RG	Urine	11.6	12.2	11.6	11.6	11.2	12.0	11.9	11.3	9.6	10.5	11.1	11.6	11.9	12.1
	Italian RG	GA + Urine	11.8	12.0	11.8	11.6	11.3	12.2	11.9	11.5	9.7	10.3	11.2	11.7	12.1	12.2
	Lucerne	Control	10.4	11.6	-	11.6	11.4	-	10.0	-	10.6	11.0	10.8	-	11.4	11.7
	Lucerne	Urine	10.5	11.6	-	11.6	11.4	-	9.9	-	10.3	10.9	10.6	-	11.1	11.7
	Lucerne	GA + Urine	10.6	11.6	-	11.6	11.4	-	9.6	-	10.2	10.7	10.6	-	11.2	11.9

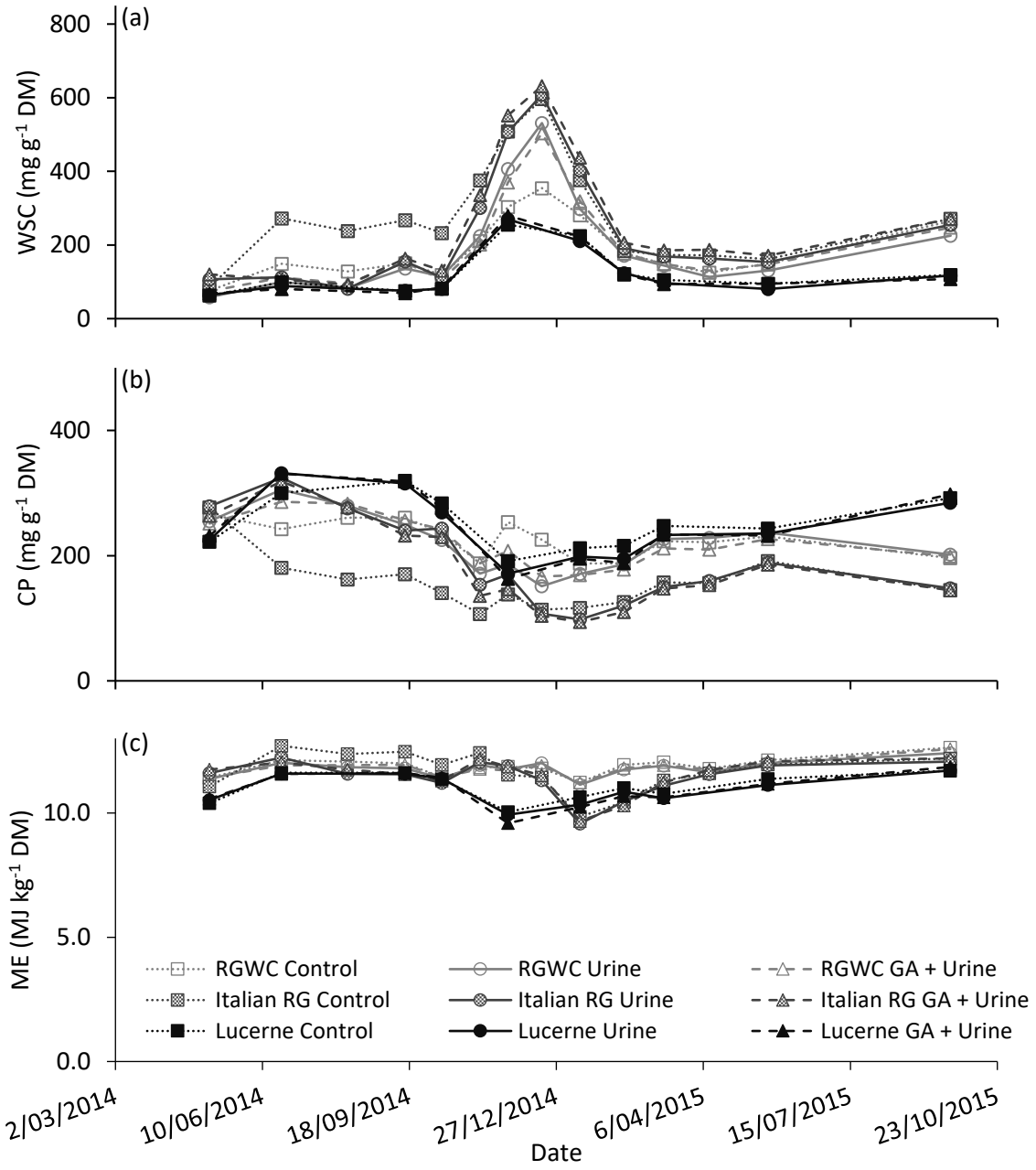

Figure C 1 Trends in forage quality parameters (mg g^{-1}): water soluble carbohydrates (WSC), crude protein (CP), and metabolisable energy (ME) (MJ kg^{-1} DM) throughout the experimental period: 7 May 2014 to 1 October 2015. Perennial ryegrass and white clover (RGWC), Italian ryegrass (Italian RG) and lucerne were treated in May 2014 with either: Control, Urine, or GA + Urine (gibberellic acid (GA) at 8 g GA ha^{-1}, urine at 700 kg N ha^{-1}).